PHYLOGENETIC RELATIONSHIPS, POPULATION CONNECTIVITY, AND THE DEVELOPMENT OF GENETIC ASSIGNMENT TESTING IN BULLER'S ALBATROSS (*THALASSARCHE BULLERI*)

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ABSTRACT

The Diomedeidae (Albatrosses) family is comprised of 22 recognised species, 13 are of high conservation concern because they are experiencing population declines. The taxonomy of albatrosses has always been problematic, which makes it difficult to estimate the number and size of breeding groups within a species. The Northern Buller's Albatross (*Thalassarche bulleri platei*) and Southern Buller's Albatross (*Thalassarche bulleri bulleri*) (Robertson & Nunn 1998; Turbott 1990) were recognised as separate species until 2006. A review of morphological data provided a basis for defining them as one species (*Thalassarche bulleri*); a result that was supported by international conservation agreements. However, there was no genetic data available at the time to corroborate the taxonomic change. The species status of as Buller's Albatross ssp. is an important issue because they are consistently recorded in the top five observed seabird interactions with commercial fishing vessels within New Zealand's Exclusive Economic Zone. Despite their prevalence in fisheries interactions, the relative impact of commercial fishing activity on northern and southern populations is unknown. Incidental mortality of albatrosses in commercial fisheries is recognised as a primary source of population disturbance.

The overall goal of this thesis research was to investigate the genetic differences between the two sub-species of Buller's Albatross. DNA was isolated from blood samples collected from a total of 73 birds from two Northern Buller's Albatross colonies (n = 26) and two Southern Buller's Albatross colonies (n = 47). The degree of genetic differentiation between the Northern and Southern taxa was estimated using DNA sequences from a 221 bp fragment of the mitochondrial control region, Domain II (CRII). The genetic differentiation between regional colony groups was high (pairwise $\Phi_{ST} = 0.621$, $p < 0.00001$). Two haplogroups were identified within Northern Buller's Albatross, while Southern Buller's Albatross samples composed a single haplogroup. An analysis of molecular variance did not find any significant population structuring at the colony level. All individuals sampled from

fisheries bycatch (n = 97) were assigned with maximum probability to either Northern (n = 19) or Southern Buller's Albatross (n = 78; P = 1.00). The DNA sequences differences found in the mitochondrial control region can be used to assign provenance of *T. bulleri* ssp. samples, which will be a useful conservation management tool.

In addition, a genome wide set of markers was obtained using a Genotyping by Sequencing approach. DNA was digested using restriction enzymes, fragments were labelled adaptor sequences, and shotgun sequenced on an Illumina platform by AgResearch. The Stacks pipeline was used to filter the sequences and obtain a set of single nucleotide polymorphism (SNP) markers across the genome. Estimates of genetic diversity and gene flow were conducted for 26 319 putative loci comprised of 54,061 single nucleotide polymorphisms. Estimates of genetic diversity were consistent across data sets with both taxa exhibiting similar levels of nucleotide diversity (Northern $\pi \approx 0.002 - 0.004$; Southern $\pi \approx 0.002 - 0.003$). However, estimates of genetic differentiation increased slightly as filtering protocols became increasingly restrictive ($F_{ST} \approx 0.019 - 0.048$). This low level of differentiation was supported by admixture analyses, which identified two distinct 'clusters', one corresponding to *T. b. platei* and the second to *T. b. bulleri*. The results of this research demonstrate that Northern and Southern Buller's Albatrosses are two genetically distinct groups.

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44	Motuhara, The Forty-fours
A	Adenine
AMOVA	Analysis of Molecular Variance
ACAP	Agreement on the Conservation of Albatrosses and Petrels
AS	Alert Stack
BAPS	Bayesian Assignment of Population Structure
BI	Broughton Island
bp	Base pairs
BSC	Biological Species Concept
C	Cytosine
COI	Cytochrome *c* oxidase, subunit I
CR	Control region
CRI	Control region, Domain I
CRII	Control region, Domain II
DNA	Deoxyribonucleic acid
EEZ	Exclusive Economic Zone
G	Guanine
GBS	Genotyping by Sequencing
GPS	Global Positioning System
H_n	Number of haplogroups
HW	Hardy-Weinberg
HWE	Hardy-Weinberg Equilibrium
IBT	Isolation-by-Time
IUCN	International Union for Conservation of Nature
k	Number of nucleotide differences
K	Number of 'clusters' or haplogroups
LS	Little Solander Island
mBSC	Multidimensional Biological Species Concept
MSC	Morphological Species Concept
mtDNA	Mitochondrial DNA

n	Number of samples
NE	North East Island
NGO	Non-governmental Organisation
NGS	Next Generation Sequencing
n_h	Number of haplotypes
NZ	New Zealand
NZPA	National Plan of Action to Reduce the Incidental Catch of Seabirds in New Zealand Fisheries
p	Probability
P	Posterior probability distribution
PCA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PSC1	Phylogenetic Species Concept 1
PTT	Platform terminal transmitter
RRL	Reduced Representation Library
S	Segregating sites
SI	Solander Island
T	Thymine
TE	Tris-EDTA solution
TK	Manawatawhi, Three Kings Islands
TS	Rangitatahi, The Sisters Islands

CHAPTER 1 GENERAL INTRODUCTION

Seabirds encompass a diverse range of species and can be found throughout the world's oceans. Yet, despite this prevalence in a range of ecosystems, seabirds are recognised as one of the most threatened groups of birds worldwide (Croxall *et al.* 2012). Of seabirds with known population trends, roughly half are undergoing decline (IUCN 2016). However, some groups are more heavily impacted than others, with pelagic taxa experiencing disproportionately high rates of decline (Croxall *et al.* 2012). Identifying and assessing the threats to seabirds is a complex and often subjective task, which can vary with ecology and life-history traits (Croxall *et al.* 2012). Unsurprisingly, taxa that forage inshore, such as Auks, Gulls (Order: Charadriiformes) and Cormorants (Order: Pelecaniformes), encounter different threats than those with pelagic foraging ranges, such as Albatrosses, Petrels and Shearwaters (Order: Procellariiformes). Procellariiform populations are particularly vulnerable to disturbances because they generally have low fecundity and a late age of sexual maturity. These life history traits hinder population recovery following a disturbance. This is an important consideration as procellariiform populations can be negatively impacted by anthropogenic activities. Fisheries bycatch has been demonstrated to be the main driver of many population declines in Procellariiformes (Croxall *et al.* 2012).

Commercial longline (Anderson *et al.* 2011; Cooper *et al.* 2001; Gilman *et al.* 2005), and trawl (González-Zevallos & Yorio 2006; Sullivan *et al.* 2006b; Thompson *et al.* 1994) fisheries often overlap with seabird foraging areas. The concentration of these vessels in important seabird areas increases the probability of interaction between fishing operations and Procellariiformes (*see* Croxall *et al.* 2012). This is compounded by the fact that these activities often attract birds to the vessels because the caught fish represent an obvious food source, particularly as they are hauled on board and processed or discarded. The majority of the interactions between vessels and birds occur when gear is being set or retrieved. Longline vessels utilise hundreds to thousands of baited hooks intermittently attached to a main line. Baited hooks can take time to

sink, enabling birds to chase the bait and become hooked. Drowning inevitably occurs when a caught bird is dragged down by the weight of the fishing gear. Trawl vessels use warp lines to set and drag nets at a specific depth in the water column. As the vessels move through the water, both warp lines and nets can strike and kill or injure birds (Sullivan *et al.* 2006b).

Bycatch Mitigation and Conservation Efforts

In response to high levels of incidental mortality in longline and trawl fisheries, some fleets have implemented mitigation measures to reduce seabird bycatch. On longline vessels, weighted hooks are used to sink the bait quickly through the turbulence caused by the moving ship before birds get a chance to give chase (*see* Melvin *et al.* 2014; Løkkeborg 2003, 2011). Scare tactics have been implemented in both longline and trawl fisheries, and appear to be effective means of reducing bird bycatch. An example of this approach is the use of Tori Lines, which are brightly coloured streamers attached to long- and warp lines. These form a physical and visual barrier to discourage birds from getting too close to gear (*see* Bull 2009; Løkkeborg 2003, 2011; Melvin *et al.* 2014; Sullivan *et al.* 2006).

Generally, mitigation practices utilise a combination of weighted lines, scaring tactics and setting/retrieving gear at night (Bull 2009; Cherel *et al.* 1996; Løkkeborg 2003; Melvin *et al.* 2014; Murray *et al.* 1993). Another important factor influencing seabird interactions is the cast off of fisheries offal and discards (Abraham *et al.* 2009; Sullivan *et al.* 2006a; Weimerskirch *et al.* 2000). The level of discharge, and the dispersal method, are known to strongly influence the rate of interactions (Sullivan *et al.* 2006a; Sullivan *et al.* 2006b; Watkins *et al.* 2008), irrespective of the presence of bird scaring lines (Abraham *et al.* 2009). As a result, the most effective primary mitigation measure is reducing the availability of offal and discards (Bull 2009; Wienecke & Robertson 2002).

The results of many studies suggest that mitigation measures will reduce incidental seabird mortality, if used properly (Brothers *et al.* 1999; Cooper *et al.* 2001; Croxall *et al.* 2012; Gilman *et al.* 2005). However, the success

of these mitigation measures in a fishery remains difficult to assess for a number of reasons. The lack of rigour in testing methodology continues to pose a challenge (Bull 2009). This is further compounded by a lack of compliance with, or proper deployment of, these measures. In a number of trials, mitigation devices did not consistently adhere to trial specifications (*see* Bull 2009). In addition, monitoring and enforcement has historically been poor (Gilman *et al.* 2005).

Prioritizing conservation management is essential for persistence of many seabird populations (Goutte *et al.* 2014; Schofield & Bond 2016; Tasker *et al.* 2000). In recognition of the threatened status of Procellariiformes, many non-governmental organisations (NGO's), such as the International Union for Conservation of Nature (IUCN) and BirdLife International, have committed to a coordinated international response. In addition, governments have ratified legislation, like the Agreement on the Conservation of Albatrosses and Petrels (ACAP), to help mitigate anthropogenic impacts on seabirds.

The IUCN is recognised as an authority on global biodiversity conservation. Established as a membership union in 1948, the IUCN works with government and civil society organisations to inform and enable conservation efforts worldwide. While the IUCN is an organisation with broad biodiversity, climate and sustainability goals, BirdLife International and ACAP focus conservation efforts on avian taxa. BirdLife International is a partnership of individual NGO's around the world. These NGO's maintain an independent identity while coordinating collaborative projects across international borders.

Finally, ACAP operates with the specific aim of mitigating known threats to Albatrosses and Petrels. Initially signed by 7 countries in 2001, ACAP has now been ratified by 13 nations and is a leading authority in procellariiform conservation. Their areas of focus include bycatch monitoring and mitigation, population monitoring, breeding site management, and taxonomic reviews. These organisations, and others like them, are helping to overcome conservation challenges by acting as a medium for international

collaborations. As a result, considerable progress has been made in addressing seabird bycatch (Croxall *et al.* 2012).

Significance of Taxonomy in Conservation

In general, how groups like ACAP, BirdLife International and IUCN characterize and distinguish species will, in turn, determine how effectively conservation agencies can organise and prioritise their efforts (Croxall *et al.* 2012). These considerations are particularly significant where the taxonomic status of a species remain contentious. Determining the number of species within the Diomedeidae (albatross) family has been difficult (*see* Abbott & Double 2003a; Alderman *et al.* 2005). Four distinct genera are universally recognised, *Diomedea*, *Phoebastria*, *Phoebetria* and *Thalassarche* (Brooke 2002; Dickinson & Remsen 2013; Gill *et al.* 2010), but, in some cases, ranks of taxa within genera have yet to reach a general consensus (*see* Burg & Croxall 2004). Furthermore, there is a lack of consensus among conservation authorities regarding diagnostic morphological traits, and inadequate genetic data (*see* Double 2006). The assignment of conservation status is determined by how a given group is defined (i.e. a distinct species, or differentiated populations). The ambiguity in the status of a number of albatross taxa has made the estimation of breeding groups challenging (Cracraft 1983; de Queiroz 2005; Mayr 1942; Mayr 1996; Zink & McKitrick 1995). Long-term monitoring of the number of breeding adults, and fledgling success provides vital information on population dynamics (Clutton-Brock & Sheldon 2010). While there is no doubt that direct monitoring of demography is useful, these studies need to be long-term in order to understand taxa where a generation time spans a decade or more (Clutton-Brock & Sheldon 2010; Lepetz *et al.* 2009; Magurran *et al.* 2010). Even in medium-term studies of long-lived birds, the dynamics of only a small number of generations may be observed and then the results incorrectly extrapolated to represent multiple generations (Magurran *et al.* 2010; Slatkin 1987). Short term studies do not provide the data necessary for 'on the ground' decision-making and can lead to incorrect assumptions and inappropriate subsequent action (Magurran *et al.* 2010).

Genetic studies can provide insights into population structure that are difficult to obtain in observational studies. Analysis of data from molecular markers can reveal patterns of gene flow and diversity. These methods identify isolated reproductive units within a species, without the need for decades of careful observation (Eda *et al.* 2008; Huyvaert & Parker 2006). Genetic markers are also a powerful tool for distinguishing whether a group is a differentiated population or distinct taxon (Aliabadian *et al.* 2009; Hebert *et al.* 2004b). The application of genetic analyses has revealed a number of 'cryptic species', i.e. species that are morphologically similar to one another, despite being genetically distinct. The identification and description of cryptic species is relatively simple if taxa are sympatric. The observed reproductive isolation may be the result of, or maintained by, pre- and post-zygotic processes preventing interbreeding. In contrast, when considering allopatric taxa, distinguishing cryptic species from genetically differentiated populations can be challenging. This is often the case in many seabird taxa where breeding colonies occur on discrete islands. This geographic separation, and natal philopatry displayed by many species, may potentially lead to the inaccurate classification of cryptic taxa.

Species delimitation from genetic data alone can be a subjective task. The observed differentiation between groups may not adhere to any one species concept as seen in the case of Albatrosses (Family: Diomedeidae). Nevertheless, when coupled with ecological and morphological data, genetic information can provide powerful insights into taxonomic relationships and population structure to identify and define priority taxa for conservation (Angelica Gutierrez-Aguirre *et al.* 2014; Bickford *et al.* 2007; Huemer *et al.* 2014).

Species Concepts in Albatross (Family: Diomedeidae) Taxonomy

Species are considered the fundamental unit of evolution and biodiversity (de Queiroz 2005, 2007; Mayr 1996). However, the conceptualisation of species and how they are defined has yet to reach a consensus. Mayden (1997) described 24 distinct species concepts, most with multiple definitions. Disagreement over theoretical species concepts has

become entangled with the issue of species delimitation, which is the determination of boundaries between species and the quantification of species from empirical data (de Queiroz 2007).

Currently, the biological species concept (BSC) is the most widely applied concept in vertebrates (Bickford *et al.* 2007). Initially introduced by Ernst Mayr (1942), the BSC identifies species as "groups of interbreeding natural populations that are reproductively isolated from other such groups." However, reproductive isolation is difficult to measure and not directly related to phenotypic and genotypic variation (Donoghue 1985). Therefore, in taxa with allopatric populations, additional data and concepts are often required to define species (Double 2006). Common alternative species concepts include the morphological species concept (MSC), and the phylogenetic species concept (PSC1); (*see* de Queiroz 2007). For birds at least, there has been a global shift away from the BSC towards the PSC1 (and related concepts), so that the number of recognised species is increasing primarily through 'taxonomic inflation' rather than discovery of species novel to the literature (*see* Gill *et al*. 2010).

Like other bird groups globally, procellariiform taxa were mainly defined using the BSC since the widespread adoption of this species definition after 1942 and morphology was taken as a proxy for inferring a biological species. Key morphological characteristics for Procellariiformes have included bill length and shape, plumage coloration, and body size (e.g., wing and tarsus length). Although morphological characters can be relied upon to discern many avian taxa, overlaps in these characteristics and disagreement on diagnostic traits can make the identification of some taxa difficult, if not impossible. Some traits may be variable for taxa at different life history stages or for a particular gender. Taxonomists must ensure that a suitable number of specimens are described to account for the full range of phenotypic variability within a taxon.

The reliance on morphological characteristics, and the strict application of species concepts, may lead to incorrect conclusions regarding species status (Bickford *et al.* 2007). Again, this can complicate estimates of breeding groups and the assignment of conservation status (Isaac *et al.* 2004). Genetic methods

are increasingly utilised to contribute additional data in order to resolve existing taxonomic challenges (Meier 2008). The stability of DNA through life stages, adherence to evolutionary models, and the ability to determine population structure allow genetic methods to clarify taxonomic relationships (Alderman et al. 2005; Milot et al. 2008; Sunnucks 2000), and investigate the evolution of diversity patterns (Blaxter 2004).

When discussing taxonomic revisions within albatrosses, I have chosen to follow the most recent naming conventions for New Zealand taxa as proposed by Gill *et al.* (2010) for simplicity and continuity and because these conventions have been widely accepted.

The challenges associated with the application of species concepts have been exemplified in the controversy surrounding the taxonomy of albatrosses. Following many years of contested taxonomic revision, Robertson and Nunn (1998) utilised genetic, morphological and behavioural data to propose that 14 traditionally recognised species be split into 24. Initially, there were concerns regarding the author's chosen Phylogenetic Species Concept (PSC1). The PSC1 (Cracraft 1983; Zink 1996, 1997; Zink & McKitrick 1995) asserts that any population that is morphologically diagnosable warrants species status. As a result, organisms with varying degrees of differentiation may all be elevated to species rank. Further, the approaches used to resolve differentiation among taxa are sometimes subjective, as different methodologies may provide variable levels of resolution (Haffer 1997). In another taxonomic review of Procellariiformes, Penhallurick and Wink (2004) suggested that the application of PSC1 by Robertson and Nunn (1998) resulted in the erroneous elevation of taxa to species status. To address this, Penhallurick and Wink (2004) utilised the same gene as Robertson and Nunn (1998), the mitochondrial gene cytochrome *b*. However, Penhallurick and Wink (2004) applied the multidimensional Biological Species Concept (mBSC) (Mayr 1996). In contrast to PSC1, mBSC asserts that species may be composed of local, and possibly temporally isolated populations that have morphologically or genetically differentiated. These populations may be part of a *polytypic species*, i.e. subspecies.

Penhallurick and Wink (2004) supported the expansion of 2 genera (*Diomedea, Phoebetria*) to four (*Diomedea, Phoebastria, Phoebetria* & *Thalassarche*) as proposed by Robertson and Nunn (1998). However, the splits between Royal Albatross (*Diomedea sanfordi* & *D. epomophora),* Wandering Albatross (*D. amsterdamensis*, *D. antipodensis antipodensis*, *D. a. gibsoni*, *D. dabbenena*, & *D. exulans*), Yellow-nosed Albatross (*Thalassarche carteri* & *T. chlororhynchos*), Black-browed Albatross (*T. impavida* & *T. melanophris*), Buller's Albatross (*T. bulleri platei*, *T. b. bulleri*), and Shy Albatross (*T. cauta cauta, T. c. steadi, T. eremita,* & *T. salvini*,) were rejected (Penhallurick & Wink 2004). However, Penhallurick and Wink's (2004) study was contentious and heavily criticised (Rheindt & Austin 2005) and the status of these taxa continued to be debated. Essentially, much of the debate revolved around the application of species concepts (*see* Rheindt & Austin 2005). Penhallurick and Wink (2004) notably commented that *D. amsterdamensis* (Amsterdam Albatross), a seemingly 'good' biological species, showed little genetic differentiation from the other proposed subspecies of Wandering Albatross. This instigated further investigation into phylogenetic relationships amongst the Wandering albatrosses. To investigate genetic differentiation among Wandering Albatross taxa, Burg and Croxall (2004) utilised sequence data from the mitochondrial control region, Domain I (Sorenson *et al.* 1999) and microsatellite markers (Burg 1999). The study included 772 individuals from all but two breeding colonies (n=7). The authors reported significant genetic differentiation amongst three out of the four proposed Wandering Albatross taxa and suggested that *D. a. antipodensis* and *D. a. gibsoni* belonged to a single species. However, if strictly adhering to the BSC, then the separation of Wandering albatrosses into four distinct, reproductively isolated species is well supported by their asynchronous reproductive patterns and isolated breeding populations (Marchant & Higgins 1990; Tickell 2000; Walker & Elliott 1999) *D. a. antipodensis* and *D. a. gibsoni* have since been recognised as subspecies by the New Zealand bird Checklist Committee (Gill *et al.* 2010) and by international agreements (CMS 2015; Double 2006). The Wandering Albatross

species complex is reflective of the piecemeal approach adopted by avian taxonomic working groups (Double 2006).

However, the taxonomic status of other albatrosses has yet to be resolved. Yet another investigation into cytochrome *b* yielded contrasting results. The study by Chambers *et al.* (2009) was the first, and remains the only study to include sequences from all 24 named albatross taxa. The authors, in keeping with conventions adopted in the Wandering Albatrosses and practiced by international conventions (Double 2006), advocated a 'total evidence' taxonomic approach. This method incorporates all available published information to determine if taxa warrant species status. Chambers *et al.* (2009) noted when implementing this approach, the broader literature supported many of the initial taxonomic splits proposed by Robertson and Nunn (1998). However, Antipodean and Gibson's Albatross (*Diomedea antipodensis antipodensis,* & *D. a. gibsoni*), as well as Northern and Southern Buller's Albatross (*Thalassarche bulleri platei* & *T. b. bulleri*) did not have evidence within the wider literature to support species rank. Nevertheless, the application of various mitochondrial and microsatellite markers would likely result in further subdivision (*see* Burg & Croxall 2001). For future examinations into the taxonomic relationships of albatrosses Chambers *et al.* (2009) highlighted the benefits of implementing barcoding methodology whereby inter- and intraspecific variation is used as a means of determining the threshold between proposed species (Meyer & Paulay 2005). This technique is promising for resolving the status of the few taxa, like Buller's Albatrosses, currently lacking much published data.

Genetic Markers

Mitochondrial DNA

Genetic methods have aided taxonomists by providing the data necessary to distinguish amongst species that would otherwise be difficult to assess. However, when incorporating genetic data into taxonomic revision, it is important to consider existing information to ensure a comprehensive

analysis and well supported designation. Genetic data is best utilised to complement existing taxonomic work, and should not entirely replace comprehensive taxonomic analysis (Hajibabaei *et al.* 2007; Meier 2008).

Mitochondrial DNA (mtDNA) is an extra-nuclear genome, and encodes the polypeptide subunits of basal membrane proteins utilised in electron transport and oxidative phosphorylation. In most vertebrate taxa, the mitochondrial genome consists of 37 functionally distinct genes, is maternally inherited and replicates autonomously. The self-replication of the mitochondrial genome allows for nucleotide variation to be assessed without the confounding effect of recombination between parental lineages, making mtDNA sequences particularly well suited for phylogenetic studies and species level resolution. In addition, mtDNA has a relatively fast pace of evolution (Brown *et al.* 1979). This has largely been attributed to two factors 1) relaxed functional constraint, and 2) a high rate of mutation (Avise 2000). The mitochondrial genome does not produce polypeptides directly involved in its own replication, or transcription. This may allow mtDNA sequences to be more flexible during translation. The high mutation rate of mtDNA has been attributed to a number of variables, such as exposure to free radicals present within the mitochondria, and a high rate of mitochondrial turnover. However, it is the lack of repair mechanisms that allow nucleotide substitutions to accumulate. These elements of mtDNA have commonly been exploited to resolve taxonomic challenges.

A DNA barcode is defined as a short DNA sequence from a standardized region of the genome. A number of barcoding genotypes compiled within a database can be utilised for species level identification. Barcoding techniques have revealed a number of cryptic species. The mitochondrial gene most commonly applied in avian taxonomy is cytochrome *c* oxidase subunit I (Baker *et al.* 2009; Hebert *et al.* 2003b; Hebert *et al.* 2004b). The application of COI barcoding was proposed as a relatively efficient and cost-effective method to differentiate taxa (Hebert *et al.* 2003a; Hebert *et al.* 2003b), and to enable taxonomists to efficiently bring attention to novel species (Hajibabaei *et al.* 2007; Hebert *et al.* 2004a; Huemer *et al.* 2014).

Species delimitation thresholds are determined under the assumption that sequence divergence between species will be greater than that within species (Moore 1995). A common method of assessing the performance of barcoding genes is by comparing inter- and intra-group variation (Meyer & Paulay 2005). If there is no overlap between the comparisons of genetic variation, then there is said to be a 'barcoding gap' and a threshold for differentiation is established for the species in question. However, in instances where inter- and intra-group variation overlap, no clear 'barcoding gap' is present, then there is said to be no support for species level resolution.

Concerns have been raised regarding the reliance upon a single mitochondrial gene for species identification. Specific concerns include the capacity to detect newly evolved species, detect hybridization or incomplete lineage sorting and the application of species delimitation thresholds (*see* Baker *et al.* 2009). To address these concerns Baker *et al.* (2009) assessed the performance of COI in avian taxa. The authors found that the 650 bp fragment of COI commonly used as a barcoding region for avian taxa was capable of resolving incomplete lineage sorting. Moreover, nuclear genes could be incorporated to resolve high female natal philopatry from selective sweeps. Also, COI barcodes were able to distinguish between closely related sister species. However, Baker *et al.* (2009) found that distance thresholds are not universally applicable across taxa; this is supported by a number of studies (*see* Hajibabaei *et al.* 2007). As an alternative to distance thresholds, Baker *et al.* (2009) proposed that coalescent-based techniques be used instead. This would enable tests of chance reciprocal monophyly and time of lineage separation. Mitochondrial DNA barcodes are capable of detecting incomplete lineage sorting and divergent, reciprocally monophyletic lineages (Baker *et al.* 2009). Yet, it remains difficult to distinguish speciation from population subdivision as a result of high female natal philopatry or regional selective sweeps. As a result, it is recommended that nuclear markers are incorporated to resolve the relative role of each in population structuring (Moore 1995).

Genotyping by Sequencing (GBS)

A common marker choice for population genetic studies is microsatellites, which are di-, tri-, or tetra nucleotide tandem repeats. These repeats may be variable within populations and between alleles within an individual. This marker type is relatively common across the nuclear genome and is known to have high rates of heterozygosity and mutation. These characteristics of microsatellites make them a useful tool as the relative allelic frequency and heterozygosity can be used to assess patterns of gene flow and the demographic histories of populations. However, the methodology used in microsatellite marker discovery limits markers to a specific repeat motif. This inherently restricts analyses of diversity to that specific, pre-defined motif despite the prevalence of microsatellites across the nuclear genome. As a result, traditional methods for microsatellite marker discovery are relatively expensive and inefficient. This limits population studies to anywhere from a few 10's to 100's of loci. While this level of genomic coverage has been informative, the application of next-generation sequencing to marker discovery has the ability to assess a significantly higher volume and range of variation. Next-generation techniques are capable of identifying not only microsatellite markers, but also single nucleotide polymorphisms (SNPs) and may be used to sequence the whole genome. This new wave of technology is thought to be superior to previous marker discovery methods (Davey *et al.* 2011; Elshire *et al.* 2011; Kumar *et al.* 2012).

Next-generation sequencing (NGS) techniques have provided a wonderful opportunity for studying genome wide variation. Rather than assessing a handful of variable sites to estimate population connectivity and structure, researchers are now able to examine thousands of loci from across the genome. This ability to assess a high volume of variable alleles allows for a refined, and high-resolution assessment of patterns of gene flow between, or within populations. In addition, these techniques can potentially be used to assess and differentiate selection from genetic drift, or identify relationships amongst SNPs and a given trait of interest (Davey *et al.* 2011; Robertson & Sawyer 1994).

One NGS method for marker discovery is Genotyping by Sequencing (GBS) (Elshire *et al.* 2011). The GBS methodology utilises restriction enzymes to cut the genome into smaller, more manageable, fragments. The reduction of the genome into fragments reduces the overall complexity of the genome as each fragment begins and ends with a known nucleotide sequence. This enables each fragment to be attached to a unique sample barcode and primer adapter (*see* Figure 1.1). As a result, a high volume of sequencing and genotyping may be conducted simultaneously across the genome.

Restriction enzymes are not limited to any specific chromosomal region or gene. This enables thousands of fragments representative of areas throughout the genome to be available for analysis. When compared with target enrichment methods, a restriction enzyme approach is generally less expensive and technically less challenging. There are four commonly utilised enzyme regimes, *Ape*KI, *Ape*KI/*Msp*I, *Pst*l, and *Pst*l/*Msp*l. Each restriction enzyme treatment will fragment the genome to varying degrees. In the absence of a reference genome, a more conservative approach would be likely. In other words, the chosen restriction regime would digest the genome into larger, rather than smaller fragments. This decreases the overall coverage of the genome, as there are fewer fragment ends available for sequencing. However, the certainty in the sequencing calls is increased. After digestion, a barcode is attached to the ends of the fragments, which are then sequenced (Figure 1.1). This ability to simultaneously discover markers and genotype individuals is an integral benefit of GBS. Genomic fragments are able to be associated with a given individual, allowing for the simultaneous discovery of markers and genotyping of individuals. Researchers are no longer limited to a given marker panel like those in traditional methods. This is the primary advantage of GBS. However, a limiting factor in the application of NGS is the complexity of data analysis. Advanced analysis pipelines are a necessity to handle the dataset generated (Torkamaneh *et al.* 2016). This is largely due to the inherent challenges posed by the sheer volume of data, sample multiplexing, and variation in fragment lengths, or read depth (Davey *et al.* 2011).

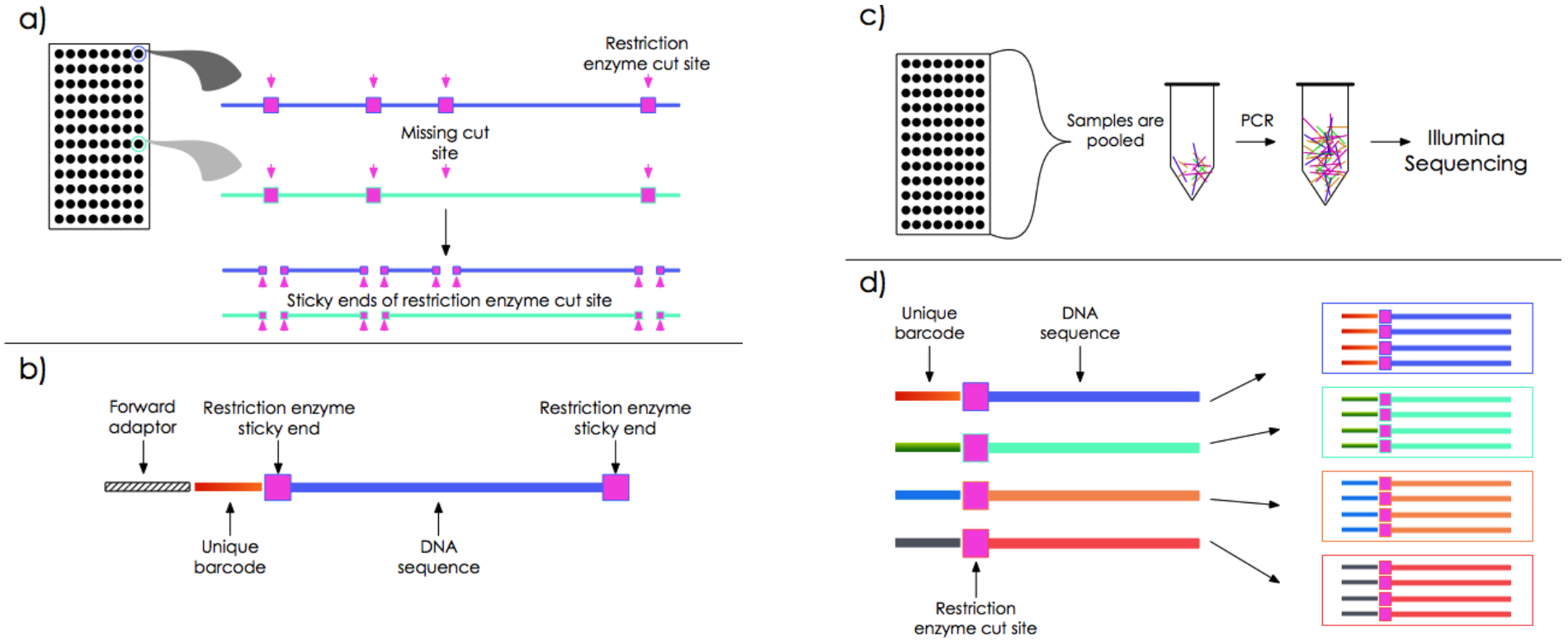

Figure 1.1 Outline of Genotyping by Sequencing methodology

a) Reduced representation libraries (RRLs) are constructed by digesting template DNA with a restriction enzyme. Cut sites may not be uniform across individuals as a result of genomic sequence variation. b) A unique 'barcode' adaptor is ligated to genomic fragments for later identification. c) The samples are then pooled together then amplified through PCR and sequenced on an Illumina platform. d) DNA barcodes are used to assign sequences to individuals. This produces a genotype to be used in analysis. Figure adapted from Myles 2013.

Study taxon: Buller's Albatross (*Thalassarche bulleri*)

The Buller's Albatross (*Thalassarche bulleri*) is a medium sized mollymawk weighing between 2.5 – 3.5 kg. The *T. bulleri* species complex is composed of two putative subspecies, Northern Buller's Albatross (*Thalassarche bulleri platei*), and Southern Buller's Albatross (*Thalassarche bulleri bulleri*). Extensive at-sea tracking data using GPS and PTT data are available for the Southern Buller's Albatross (Figure 1.2). However, there is essentially *nil* tracking data available for the northern taxon (BirdLife International 2004). Nevertheless, it is believed both taxa share feeding grounds off the coast of Chile and Peru during the non-breeding seasons (BirdLife International 2004).

Northern Buller's albatrosses return to their breeding grounds annually in September and October, whereas Southern Buller's Albatrosses return three months later in December and January. The northern taxon largely breeds on small islands around the Chatham Island's group, with a minute breeding population at the Three Kings Islands (Fraser *et al.* 2008; Fraser *et al.* 2009; McCallum *et al.* 1985; Robertson & Sawyer 1994; Scofield *et al.* 2007; Wright 1984). In contrast, the Southern Buller's Albatross nests on Solander Island and The Snares, with some birds nesting within densely wooded vegetation (*see* Figure 1.3; Robertson 1991; Sagar *et al.* 1999; Turbott 1990). This is unusual, as most albatrosses prefer open areas where they are able to easily take to wing. During the breeding season, the southern taxon may be found foraging off the coasts of mainland New Zealand and are observed further abroad near southeast Australia, and Tasmania (*see* Figure 1.2; Gill *et al.* 2010; BirdLife International 2004). Anecdotal evidence suggests that the northern taxon is found primarily along the Chatham Rise. The average age of first reproduction is 12 years for the southern taxon (Sagar & Warham 1998), and is unknown for the northern taxon. Buller's Albatrosses form monogamous pair bonds and invest heavily in parental care over the course of eight months to raise a single altricial chick. This relatively late age of sexual maturity and low fecundity

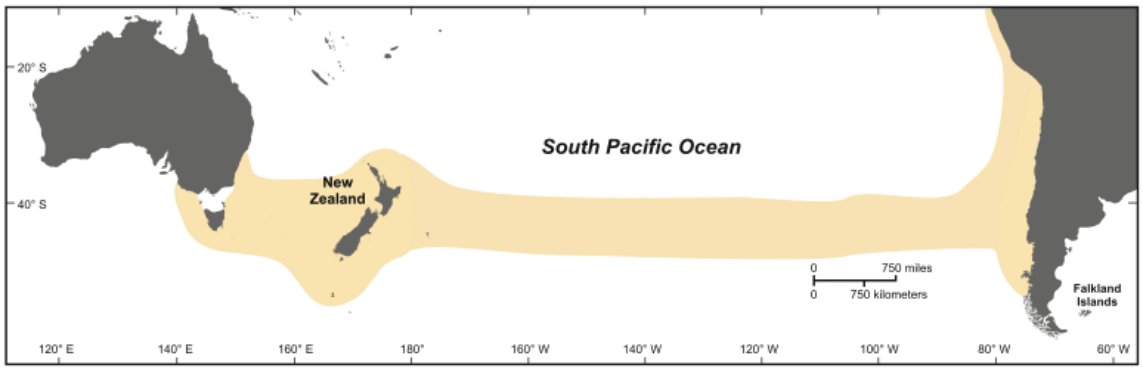

Figure 1.2 At sea distribution for Southern Buller's Albatross
Map depicts at sea distribution of Southern Buller's Albatross as derived from the Tracking Ocean Wanderers project (BirdLife International 2004; Broekhuizen *et al.* 2003; Deppe 2008; Sagar & Weimerskirch 1996; Stahl & Sagar 2000a, b).

make these populations susceptible to disturbances and may inhibit population recovery.

Since 2002, Buller's Albatross ssp. have been consistently recorded among the top five observed seabird interactions with commercial fishing vessels within New Zealand's Exclusive Economic Zone (EEZ), and are a significant source of seabird bycatch (Abraham & Thompson 2012; Clemens-Seely *et al.* 2014a, b; Clemens-Seely & Osk Hjorvarsdottir 2016). As with other albatrosses and petrels, fisheries interactions occur when birds are attracted to fishing vessels by fisheries discards and offal. In addition, the available tracking data show high overlap between Southern Buller's Albatross feeding grounds and high fishing effort. Buller's Albatross ssp. are commonly killed in longline and trawl fisheries (Abraham & Thompson 2012; Clemens-Seely *et al.* 2014a, b; Clemens-Seely & Osk Hjorvarsdottir 2016). Regrettably, it is currently difficult to identify distinguishing morphological characters between Northern and Southern Buller's albatrosses with these bycatch specimens. This is particularly true at sea, when plumage is wet. Difficultly in accurate identification and the lack of regular population monitoring of Northern Buller's albatrosses make it impossible to determine the relative impact of fisheries interactions on the two taxa.

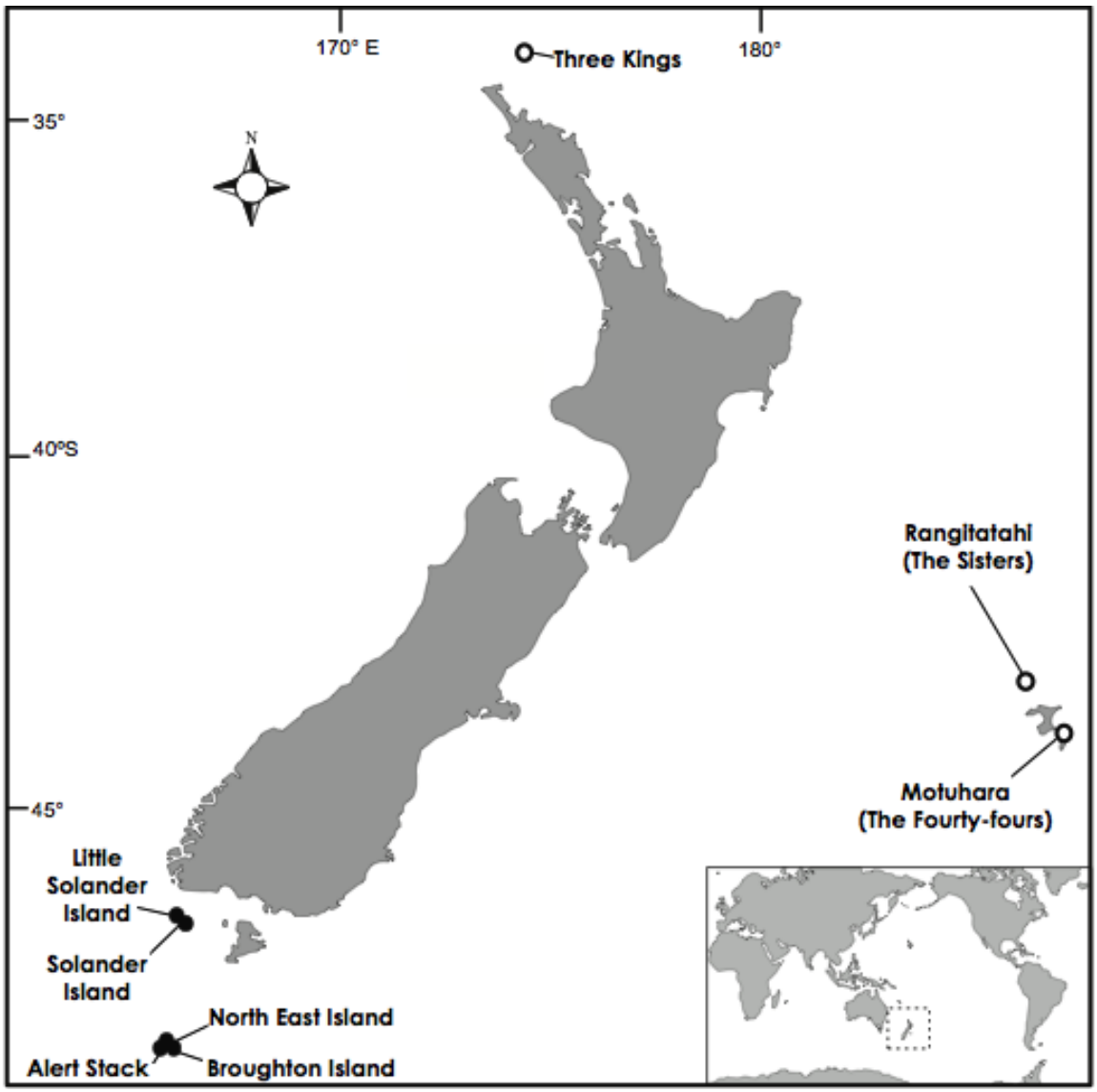

Figure 1.3 Locations of Northern and Southern Buller's Albatross breeding colonies

Locations of Northern (white circle), and Southern (black circle) Buller's Albatross colonies.

Thesis Aims and Structure

Accurate identification of Northern and Southern Buller's albatrosses through morphological methods is challenging; further it is unknown if the two groups are reproductively isolated. The specific aims of this research are to: 1) identify and utilise genetic markers to determine the degree of differentiation between the Northern and Southern Buller's Albatross, 2) to determine population structure between the two groups, and 3) to develop a method for determining the provenance of individuals.

Chapter two presents the application of a 221 bp fragment of the mitochondrial control region, Domain II to resolve the degree of genetic differentiation between Northern and Southern Buller's Albatrosses, and assign Buller's Albatross ssp. caught in fisheries to these different forms. Chapter three describes the application of GBS to further examine the degree of genetic connectivity between the Buller's Albatross ssp. Chapter four presents a summary and general discussion on the main findings of this thesis.

CHAPTER 2 GENETIC DIFFERENTIATION AND POPULATION ASSIGNMENT OF BULLER'S ALBATROSS (*THALASSARCHE BULLERI*)

Abstract

Commercial fishing activities have been linked to seabird population declines, particularly within the Diomedeidae (Albatross) family. Between 2002 and 2011, the two protected sub-species of Buller's Albatross (*Thalassarche bulleri platei* and *T. b. bulleri*) accounted for 34% of albatross interactions in the New Zealand trawl fisheries. However, determining the proportion of each taxa caught in a particular fishery is confounded by the difficulty of properly identifying the two taxa using morphological characteristics. To address this problem, the aim of this research was to develop a genetic identification method for the two taxa. DNA was isolated from blood samples collected from a total of 73 birds from two Northern Buller's Albatross (*T. b. platei*) colonies (n = 26) and two Southern Buller's Albatross (*T. b. bulleri*) colonies (n = 47). The degree of genetic differentiation between the northern and southern taxa was estimated using DNA sequences from a 221 bp fragment of the mitochondrial control region, Domain II (CRII). There was an average of 4.7% DNA sequence difference between the subspecies. However, the larger range of pairwise differences within *T. b. platei* (0 to 6.4%) obscured a clear gap in inter- and intra-taxa comparisons. Despite this large intra-taxa variation, the genetic structure of regional colony groups was high (pairwise Φ_{ST} = 0.621, p < 0.00001). Haplogroups were defined using a Bayesian assignment method and a median joining haplotype network. Two haplogroups were identified for samples of Northern Buller's Albatross, while Southern Buller's Albatross samples composed a single haplogroup. An analysis of molecular variance did not find any significant population structuring at the colony level. All individuals sampled from fisheries bycatch (n = 97) were assigned with maximum probability to either Northern (n = 19) or Southern Buller's Albatross (n = 78; P = 1.00). Neutrality tests suggested

that Northern Buller's albatrosses have a relatively stable demographic history, while Southern Buller's albatrosses are likely to have undergone a recent population expansion. This study showed that sequences from the mitochondrial control region could be used to assign Buller's Albatross ssp. caught in New Zealand fisheries to their northern and southern taxon. These results can be used for management of the subspecies and to enhance future taxonomic review.

Introduction

Tube-nosed seabirds (Order: Procellariiformes) are regarded as one of the most threatened avian orders (Croxall *et al.* 2012). Their vulnerability to disturbances is largely attributed to slow breeding rates because of long generation times and low fecundity (Arnold *et al.* 2006; Baker *et al.* 2007; Burg 2007; Croxall *et al.* 2012). Among procellariiform families, the Albatrosses (Diomedeidae) are considered a high conservation priority (Baker & Gales 2002; Croxall *et al.* 2012). Currently, 14 of the 22 recognised species are reported to have decreasing or unknown population trends by the International Union for Conservation of Nature (IUCN). The commercial fishing industry has a significant role in the population declines of some albatrosses (Lewison & Crowder 2003; Tuck *et al.* 2001; Zador *et al.* 2008). However, determining the relative impact of fishing-related mortality on a particular species or subspecies of albatross can be challenging. Especially when there is difficulty in distinguishing between morphologically similar species. This problem is often compounded by the general lack of identification and taxonomic expertise among on-board observers. Moreover, specimens can be damaged to such an extent that diagnostic traits are unrecognisable (Edwards *et al.* 2001a).

To help resolve these uncertainties, DNA-based methods have several advantages, which include not requiring well-preserved, whole specimens and being a stable characteristic throughout the entire life of an individual (Aliabadian *et al.* 2009; Hajibabaei *et al.* 2007). Genetic data has demonstrated its usefulness as a tool for identifying illegally trafficked animal products (Dawnay *et al.* 2007), and to assign provenance to species

from highly structured populations (Abbott *et al.* 2006; Burg 2007; Moritz 1994).

The concept to utilise genetic data to estimate the relative proportion of Procellariiformes represented in bycatch has been introduced relatively recently (Edwards *et al.* 2001b). For example, the Western & Central Pacific Fisheries Commission developed an assay based on restriction fragment length polymorphisms (RFLP) to identify species involved in incidental bycatch (Inoue *et al.* 2015). However, RFLPs do not have the level of resolution required to discern differences among sub-species or populations (Inoue *et al.* 2015). This has meant that the relative impacts of fisheries interactions on a particular population or group of breeding colonies are unknown (*see* Abbott *et al.* 2006; Baker *et al.* 2007; Gomez-Diaz & Gonzales-Soli 2007; Walsh & Edwards 2005 *for partial examples*). A better level of resolution can be obtained using DNA sequence data from highly variable gene regions, such as the mitochondrial genome and nuclear DNA microsatellites. A number of studies have had success with the mitochondrial control region in distinguishing population level differences within albatrosses (Abbott *et al.* 2006; Burg 2007; Walsh & Edwards 2005).

The Buller's Albatross is a species that is consistently recorded in the top five seabird interactions within the New Zealand EEZ (Abraham & Thompson 2012; Clemens-Seely *et al.* 2014a, b; Clemens-Seely & Osk Hjorvarsdottir 2016), but there is uncertainty about the status of sub-species. Northern (*Thalassarche bulleri platei*) and Southern (*T. b. bulleri*) Buller's Albatross are typically recognised as sub-species by international conservation organisations (Double 2006). However, Robertson & Nunn (1998) have proposed that *Thalassarche bulleri* be split into two distinct species. It is believed that the two taxa may be distinguished based on their plumage (Dickinson & Remsen 2013; Gill *et al.* 2010). Moreover, there have been substantial problems scoring these characteristics in poor quality bycatch specimens, making it difficult to properly assess the relative impact of fishing-related mortality on each taxon. The existing one-species classification, coupled with the cost and difficulty in accessing breeding colonies, has resulted in a low

population monitoring effort for Northern Buller's Albatross, with the last count taking place in 2009 (Fraser *et al.* 2009). In contrast, annual population counts are carried out on the Snares, the largest colony of Southern Buller's Albatross (*see* Table 2.1; Sagar 2015; Thompson *et al*. 2016). The level of genetic differentiation between the two taxa has received limited treatment. van Bekkum (2004) found differences between the groups based on data from two microsatellite DNA loci, but an analysis of the mitochondrial DNA cytochrome *b* gene appeared to show that the two taxa were conspecific (Chambers *et al.* 2009). The findings in both studies were accepted as only being provisional because of the small sample sizes for Northern Buller's Albatross (n = 7 van Bekkum 2004, n = 1 Chambers *et al.* 2009).

The aim of this study was to use DNA sequences from the mitochondrial DNA control region to assess a larger sample size to determine the phylogeographic structure of Northern and Southern Buller's Albatross populations. The hypervariable mitochondrial DNA control region, Domain II (CRII) was used because it is known to have a higher level of variation compared to other mtDNA genes. The analysis of the mtDNA sequence data will be used to 1) determine levels of genetic differentiation within and between samples from populations of the northern and southern taxa, and 2) assess whether this marker can be used to assign individual bycatch to sub-species and population of origin.

Table 2.1 Estimated colony sizes

Current estimation of contemporary colony sizes, for Northern Buller's Albatross (*Thalassarche bulleri platei*) and Southern Buller's Albatross (*T. b. bulleri*). Colony size is the estimated number of individuals breeding at each colony (Fraser *et al.* 2008; Fraser *et al.* 2009; Gales 1998; Sagar 2014; Scofield *et al.* 2007; Thompson *et al.* 2016; Wright 1984). Estimates were made with nest counts, where one occupied nest was assumed to equal a breeding pair.

Locality	Site Code	Geographic Coordinates	Colony Size (Individuals)	Taxa	Population Total
Motuhara (The 44's)	44	43°57'S, 175°50'W	29,348	Northern Buller's Albatross	33,660
Rangitatahi (Little Sister, Sisters Island Group)	TS	43°34'S, 176°48'W	4,300		
Manawatawhi (Three Kings Island Group)	TK	34°09'S, 172°08'E	12		
Alert Stack (The Snares Island Group)	AS	48°02'S, 166°34'E	610	Southern Buller's Albatross	23,028
Broughton Island (The Snares Island Group)	BI	48°02'S, 166°37'E	1,314		
North East Island (The Snares Island Group)	NE	48°01'S, 166°36'E	15,484		
Solander Island	SI	46°34'S, 166°53'E	5,280		
Little Solander Island	LS	46°34'S, 166°51'E	340		

Methods

Sampling

A total of 107 blood samples were obtained from chicks and nesting adults between 1996 and 2007 during nest count surveys. Thirty-one samples are representative of Northern Buller's Albatross (22 = Motuhara, 4 = Rangitatahi) and 76 samples are representative of Southern Buller's Albatross (49 = North East Island, 27 = Solander Island). Blood samples were stored in ethanol and kept at -4°C prior to DNA extraction. In addition to the 107 samples of known provenance, liver samples from 97 individuals were harvested during routine necropsy of bycatch between July 1999 and June 2016 (*see* Appendix I). All individuals were collected from within New Zealand's EEZ.

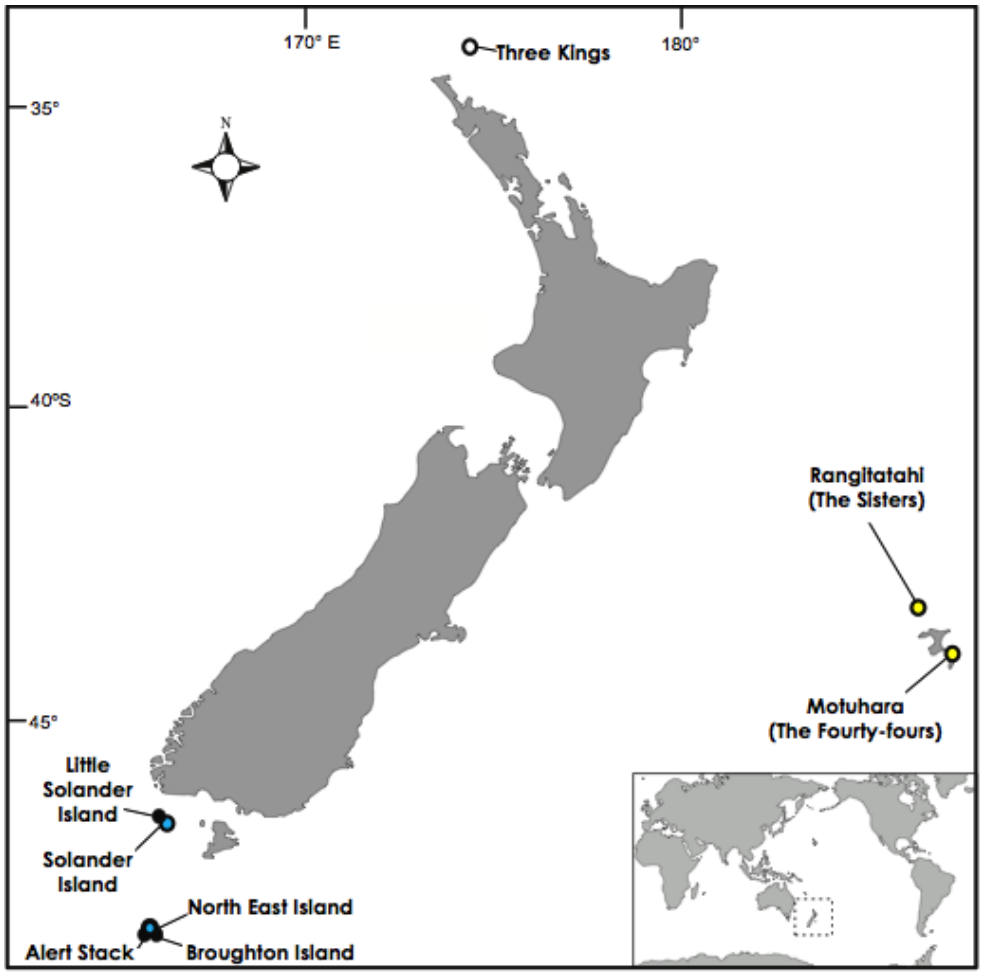

Figure 2.1 Sampled colony locations
Locations of Northern, and Southern Buller's Albatross colonies. Colonies from which blood samples were collected are denoted by yellow for Northern Buller's colonies, while blue denotes Southern Buller's Albatross colonies. Colonies not represented in this study are either white for Northern Buller's Albatross, or black for Southern Buller's Albatross. Details of localities are provided in Table 2.1.

DNA extraction, PCR amplification, and DNA Sequencing

Blood and tissue samples were digested in extraction buffer (10 mM Tris pH 8.0, 50 mM NaCl, 10 mM EDTA, and 0.2% SDS) with 0.5-µg/µL proteinase-K. The DNA was extracted using phenol and chloroform solutions (Sambrook et al. 1989). Total DNA was precipitated using ethanol, dried, and re-suspended in 30 µL TE buffer (10mM Tris. pH 8.0, 1mM EDTA). The purified DNA was stored at 4°C prior to analysis and archived at -20°C. The forward primer SPECF1 (5'-AACAGCCTATGTGTTGATGT-3') and reverse primer GluR7 (5'- CGGGTTGCTGATTTCTCG-3') from Abbott *et al.* (2005) were used to amplify a 221 bp fragment from the mitochondrial DNA control region, Domain II (CRII). PCRs consisted of approximately 50 ng of DNA, 670 mM Tris-HCl, 160 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, 0.4 µM of each primer, 0.2 µM of each dNTP, 0.05 U *Taq* polymerase, and 0.4 mg mL^{-1} of bovine serum albumin made to a total volume of 25 µL. Thermal cycling was performed on an Eppendorf Mastercycler EP Gradient S using the following conditions: 2 min at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 60°C, and 60 sec at 72°C; a final 72°C extension was carried out for 7 min. A dilution of ExoSAP-IT was used to prepare the amplified PCR products (0.5 µL ExoSAP-IT, 0.5 µL of 160mM $(NH_4)_2SO_4$, 670mM Tris-HCl) for cycle sequencing. An ABI3730 Genetic Analyser was used to determine the DNA sequence of the amplified products (Macrogen Inc., Seoul, South Korea). Initially, DNA sequences were obtained in both directions. However, after consistently obtaining high quality consensus sequences, only the primer SPECF1 was for sequencing the remaining PCR products.

Statistical Analyses

DNA sequences were aligned with CLUSTAL *W* (Thompson *et al.* 1994) and percent pairwise differences between individuals was estimated in Geneious v8.1.7 (Kearse *et al.* 2012). The relative frequency of percent pairwise differences was used to investigate fixed differences between the two groups (Meyer & Paulay 2005). The most appropriate nucleotide substitution model for this fragment of CRII was identified through likelihood ratio tests conducted in jModelTest v2.1.10 (Darriba *et al.* 2012).

To determine if the sample sizes in this study fully represent haplotype diversity within Northern and Southern Buller's Albatross, a rarefaction analysis was conducted in Analytic Rarefaction v2.1 (Holland 2012, www.huntmountainsoftware.com). DNASP v5.0 (Librado & Rozas 2009) was used to calculate haplotype diversity (*h*), nucleotide diversity (π), nucleotide differences (*K*) and the number of segregating sites (S). ARLEQUIN v3.5 (Excoffier & Lisher 2010) was used to calculate analysis of molecular varian (AMOVA), pairwise Φ_{ST} & F_{ST}, and neutrality statistics. In the original paper, Excoffier *et al.* (1992) utilised Φ to refer to the use of genetic distance or haplotype frequency interchangeably in calculating traditional F-statistics. To reduce confusion, I will use Φ to indicate the use of genetic distance, and F to designate the use of haplotype frequency in calculations. Incorporating genetic distance measures into Φ-statistics is recommended as it not only considers frequency but the number of mutational steps among haplotypes (Excoffier *et al.* 1992; Slatkin 1987). This, coupled with the application of haplotype frequency to calculations, can provide insights into patterns of gene flow. The use of haplotype frequency is a relative measure of variation as it does not consider the absolute genetic distance among haplotypes. For example, if two populations did not share haplotypes, yet the frequency of haplotypes within each population were similar, an AMOVA calculated with genetic distance will indicate different degrees of structuring than an AMOVA calculated with haplotype frequency.

Hierarchical AMOVA were used to assess variability between Northern and Southern taxa (Φ_{CT}), between breeding colonies within a taxon (Φ_{SC}), and within breeding colonies (Φ_{ST}). Because bycatch samples could not be assigned to specific colonies, only samples of known provenance were utilised to calculate hierarchical AMOVA. One AMOVA was run using genetic distance, and a second AMOVA was performed with haplotype frequencies. To further investigate differentiation, pairwise Φ_{ST} and pairwise F_{ST} were calculated on both the colony and regional level; where Motuhara and Rangitatahi represented Northern Buller's Albatross, while Solander Island and North East Island represented Southern Buller's Albatross. Finally, to assess the

potential influence of increased sample size on levels of differentiation among regions, regional pairwise Φ_{ST} and F_{ST} values were calculated again with assigned bycatch. Significance values for the AMOVA, colony and regional pairwise Φ_{ST} & F_{ST} comparisons were determined through random permutation procedures (10,000 permutations).

To investigate the population structure of Buller's Albatross without a priori assignment, data from all samples of known provenance were pooled intoa single population. Groups were identified with BAPS v6.0 (Bayesian Analysis of Population Structure; as per Corander and Tang 2007; Corander *et al.* 2008; Maltagliati *et al.* 2010). To identify the maximum number of clusters (K) and to account for the possibility of multiple clusters within regions, five replicates were run for each value of K = 1 to K = 6. Analyses were run under *cluster with linked loci* option as if for 170 reference individuals and for 500 iterations per individual. For assignment of bycatch samples, the sample sets of known provenance and bycatch were pooled into a panmictic group, and a BAPS analysis was repeated under the described conditions. Relationships among mitochondrial lineages were estimated through a median joining haplotype network (Bandelt *et al.* 1999) as implemented in PopART (Population Analysis with Reticulate Trees, http://popart.otago.ac.nz). This method of network construction builds upon Kruskal's (1956) minimum spanning network algorithm by incorporating a maximum-parsimony heuristic algorithm (Farris 1970) and a bias towards short connections. Clusters identified with BAPS in samples of known provenance, were then used to visualise the relationships among regionally unique haplogroups with the haplotype network.

Results

Known Provenance

From the 107 samples of known provenance, PCR-products were successfully obtained from 73 individuals. Of which, 26 were of Northern Buller's Albatross and 47 of Southern Buller's Albatross (*see* Table 2.2). The most appropriate substitution model identified by jModelTest was

Jukes - Cantor (Jukes & Cantor 1969). All calculations which incorporated distance measures were run under this model. Percent pairwise differences among samples of known provenance ranged from 0 to 6.4% within the Northern group, 0 to 3.6% within the Southern group, and 1.4 to 6.8% between the two (Figure 2.2). In samples of known provenance, Tajima's D was negative, but insignificant across both Northern and Southern regions. In contrast, Fu's FS was negative and significant ($p < 0.00001$) for both regions, with values higher within Southern Buller's Albatross (Fu's F_S = -23.531) than Northern Buller's Albatross values (Fu's F_S = -12.491; *see* Table 2.2).

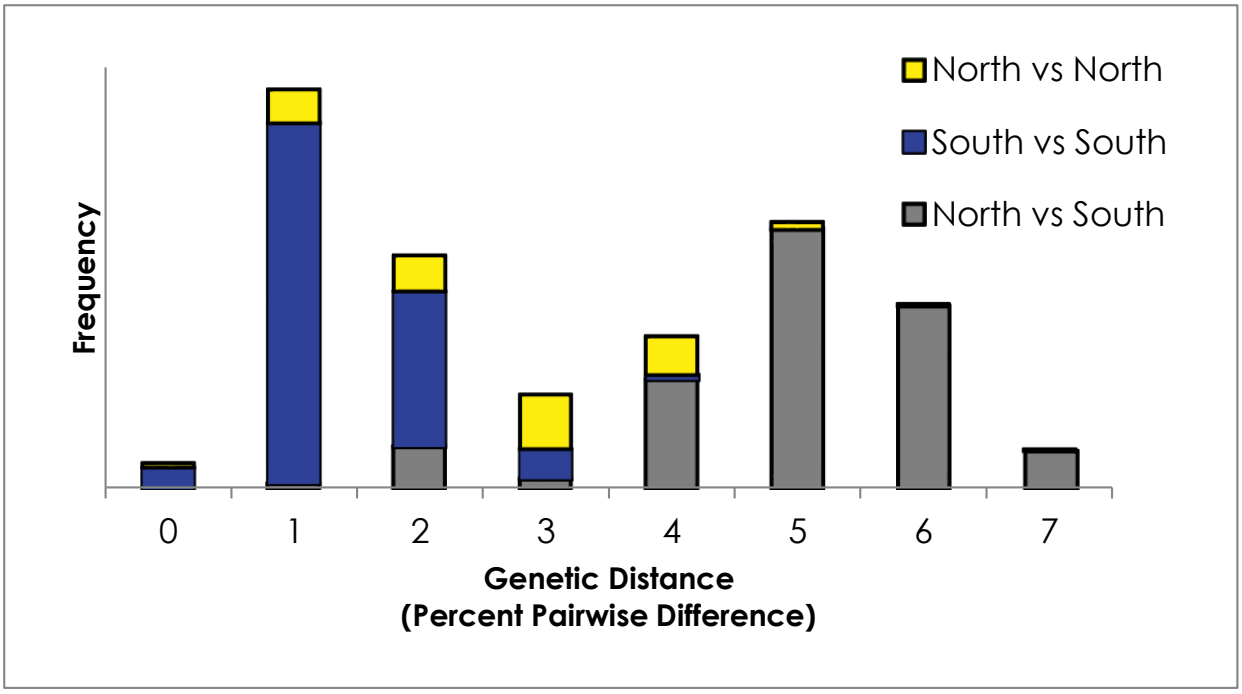

Figure 2.2 Inter- and Intra-taxa pairwise differences
Frequency of percent pairwise differences within Northern and Southern Buller's Albatross, and between the two taxa.

Table 2.3 Colony pairwise Φ_{ST}

Pairwise comparison matrix of Φ_{ST} among Buller's Albatross colonies using Jukes-Cantor genetic distance (Jukes & Cantor 1969). Motuhara and Rangitatahi are Northern Buller's Albatross colonies (*T. b. platei*), while Solander Island and North East Island are Southern Buller's Albatross colonies (*T. b. bulleri*).

	Motuhara	Rangitatahi	Solander Island
Motuhara	-		
Rangitatahi	0.0917 ^{NS}	-	
Solander Island	0.6069***	0.7032***	-
North East Island	0.5863***	0.6629**	-0.0094 ^{NS}

^{NS} Nonsignificant, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.00001$

Table 2.2 Genetic diversity indices and neutrality statistics from CRII sequences from Buller's Albatross ssp. (*Thalassarche bulleri* ssp.) of known provenance

N, sample size; *S*, number of segregating sites; n_h, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity; *K*, nucleotide differences.

Sample Information				Diversity Indices					Neutrality Statistics	
Region	**Colony**	**Site Code**	**N**	**S**	**n_h**	***h***	**π**	***K***	**Fu's F_S**	**Tajima's D**
Northern			26	29	21	0.982	0.026	5.726	-12.491***	-0.913^{NS}
	Motuhara	44	22							
	Rangitatahi	TS	4							
Southern			47	21	27	0.963	0.013	2.931	-23.531***	-1.243^{NS}
	North East Island	NE	24							
	Solander Island	SI	23							

^{NS} Nonsignificant, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.00001$

Table 2.4 Analysis of Molecular Variance
Φ - statistics for CRII from *Thalassarche bulleri* conducted on geographically structured samples of known provenance. Analyses were run under Jukes-Cantor distance measures (Jukes & Cantor 1969).

Source of Variation	D.F.	Sum of Squares	Percentage of Variance	Φ-statistics
Among Regions	1	109.924	61.069	$\Phi_{CT} = 0.6038^{NS}$
Among Breeding Colonies within Regions	2	5.990	1.31	$\Phi_{SC} = 0.0330^{NS}$
Within Breeding Colonies	69	135.317	37.63	$\Phi_{ST} = 0.6237^{***}$

^{NS} Nonsignificant, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.00001$

Colony pairwise Φ_{ST} values demonstrated high levels of differentiation between colonies from Northern and Southern regions (Table 2.3). Yet, comparisons between colonies within regions were insignificant. The largest significant pairwise Φ_{ST} value was found between Rangitatahi and Solander Island (pairwise $\Phi_{ST} = 0.703$, $p < 0.00001$). All comparisons between colonies should be viewed with caution due to the small sample sizes, particularly comparisons including Rangitatahi (n = 4). Regional pairwise Φ_{ST} values among samples of known provenance showed a high degree of differentiation and were significant (Regional pairwise $\Phi_{ST} = 0.618$; $p < 0.00001$). In contrast, regional pairwise F_{ST} values revealed low, but significant, levels of differentiation.

AMOVA calculated using both Jukes-Cantor distance (Jukes & Cantor 1969) and haplotype frequency identified no significant regional differentiation and no significant level of differentiation between breeding colonies within a region. Because both AMOVA returned similar results, only the results from the AMOVA calculated with Jukes-Cantor distance are reported here (Table 2.4). While both AMOVA indicated that the observed variation was found within the breeding colonies, it is important to note that the AMOVA calculated with haplotype frequency returned overall lower values and indicated that there was little, but significant, variation within breeding colonies ($F_{ST} = 0.027$, $p < 0.05$; *see* Appendix II). In contrast, the AMOVA calculated with Jukes-

Cantor distance indicated high variation within breeding colonies ($\Phi_{ST} = 0.624$, $p < 0.00001$).

A total of three haplogroups were identified from the samples of known provenance using BAPS. Haplogroups I & II were exclusive to Northern individuals (n = 24) and haplogroup III was exclusive to Southern individuals (n = 47). One haplotype, shared by two individuals collected from the Northern Motuhara colony, could not be assigned to one of the three identified haplogroups ($P < 0.001$). However, BAPS revealed that this particular haplotype was most similar in sequence to Haplogroup III.

The haplotypes for each region present differing patterns within the network (Figure 2.4). Overall the haplotypes observed in Northern Buller's albatrosses tended to be at a low frequency compared to the haplotypes observed in Sothern Buller's albatrosses, and multiple mutational steps separated several Northern haplotypes. In contrast, there were generally fewer mutational steps between haplotypes within the Southern group, which showed a starburst-like pattern (Slatkin & Hudson 1991). The two Northern individuals sharing a haplotype that was not assigned to a haplogroup in BAPS can considered an intermediary DNA-sequence type. This haplotype shows a close relationship with all three haplogroups. Though fewer mutational steps separate this haplotype from haplogroup III than from haplogroups I & II.

Bycatch Inclusive

All 97 samples collected from bycatch specimens were successfully sequenced. For the dataset, the rarefaction analysis did not show a trend towards a flattening slope (*see* Appendix II). If the sampling effort adequately represents the level of diversity for each population the trend line would plateau as increased sampling effort reveals fewer novel haplotypes. The datasets for both Northern and Southern Buller's Albatross appear to be under-sampled.

After the inclusion of assigned bycatch, there were minor changes to diversity indices. The number of segregating sites, haplotypes, and nucleotide differences increased for both Northern and Southern taxa. While nucleotide

Table 2.5 Genetic diversity indices and Neutrality Statistics from CRII sequences from Buller's Albatross of known provenance and assigned bycatch

N, sample size; *S,* number of segregating sites; n_h, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity; *K*, nucleotide differences

Sample Information		Diversity Indices					Neutrality Statistics	
Region	***N***	***S***	$\mathbf{n_h}$	***h***	**π**	***K***	**Fu's F_S**	**Tajima's D**
Northern	45	36	31	0.976	0.026	5.769	-20.393***	-1.026^{NS}
Southern	125	36	57	0.962	0.014	3.109	-26.354***	-1.613*

^{NS} Nonsignificant, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.00001$

diversity did not change for the Northern taxa, it underwent a slight increase from 0.013 to 0.014 in the Southern taxa. In contrast, haplotype diversity decreased in both groups from 0.982 to 0.976 for Northern Buller's Albatross and from 0.963 to 0.962 for Southern Buller's Albatross. Tajima's D remained insignificant for the Northern taxon, but was significant for the Southern taxon. Fu's F_S remained significant for both groups and increased from -12.491 to –20.393 in Northern Buller's Albatross and from –23.531 to -26.354 in Southern Buller's Albatross (Table 2.5). When bycatch samples were assigned to their region of origin and included in the calculation of regional pairwise Φ_{ST}, values remained significant and increased from 0.618 to 0.621 ($p < 0.00001$). Regional pairwise F_{ST} also increased with the inclusion of bycatch from 0.027 to 0.031 ($p < 0.00001$).

The three haplogroups identified in samples of known provenance were retained after the inclusion of the 97 bycatch samples to the BAPS analysis. All 97 individuals were assigned to one of these three haplogroups with maximum probability ($P = 1.00$). Of the 97 samples of unknown provenance, 19 belonged to haplogroups identified in Northern Buller's Albatross and the remaining 78 belonged to the Southern Buller's Albatross haplogroup. Yet, the previously discussed ambiguous Northern Buller's Albatross haplotype remained unresolved.

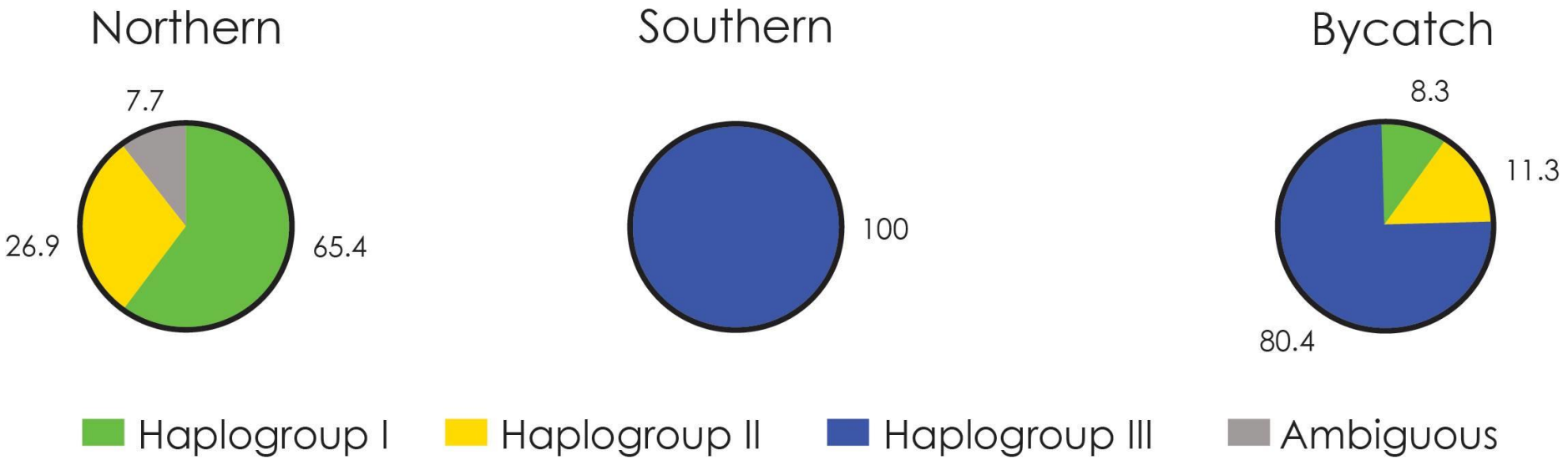

Figure 2.3 Proportion of assignment identified in BAPS

This figure demonstrates the proportion of CRII haplogroups identified in Buller's Albatross samples of known and unknown origins. Northern Buller's Albatross is composed of two haplogroups. Two samples sharing the same haplotype, and from the same island, returned ambiguous results. In contrast, all Southern Buller's Albatross individuals were assigned to Haplogroup III. Of the individuals caught as bycatch, a majority (80.4%) were assigned to Southern Buller's Albatross. The remaining 19.6% *of* individuals were determined to belong to Northern Buller's Albatross haplogroups (19.6%). All bycatch were assigned with maximum probability ($P = 1.00$).

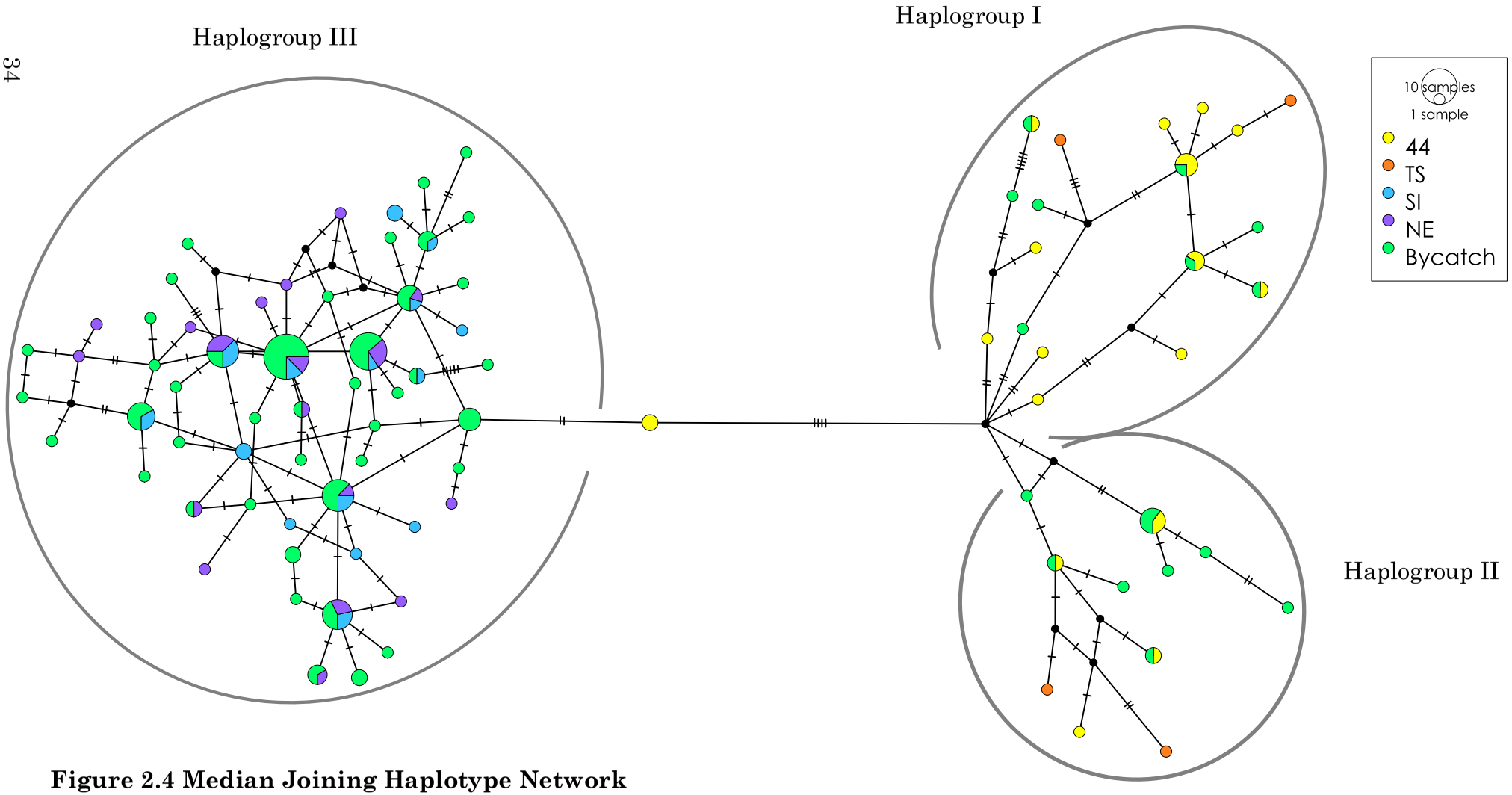

Figure 2.4 Median Joining Haplotype Network

Median joining haplotype network constructed from a 221 bp fragment of CRII for 45 Northern Buller's Albatross, and 125 Southern Buller's Albatross. Each circle represents a distinct haplotype; the relative size of each represents the number of individuals sharing that haplotype. Hash marks indicate one mutational change. Each colour is representative of a sample's origins: 44, Motuhara; TS, Rangitatahi; SI, Solander Island; NE, North East Island; Bycatch, individuals collected as fisheries bycatch from the EEZ.

Discussion

The successful assignment of all bycatch samples to their population of origin (Figure 2.3) strongly supports the value of CRII as a genetic marker for identifying and assigning birds to their subspecies group. Of the bycatch, 20% were assigned to haplogroups associated with Northern Buller's Albatross. Despite the lack of a 'barcoding gap', the haplotype network and BAPS analysis indicated that Northern and Southern Buller's Albatrosses are comprised of distinct mitochondrial lineages (Figure 2.3 & Figure 2.4). The haplotype network patterns and neutrality statistics suggest the Northern Buller's Albatross population has been demographically stable. In contrast, the starburst pattern of haplotypes and negative neutrality statistics suggests that the Southern Buller's Albatross has undergone a population expansion. The finding of an expanding Southern population is further supported by the lower haplotype diversity, nucleotide diversity, and nucleotide differences, both with and without bycatch samples.

Genetic Diversity

The present study is the first to incorporate a sufficient sample size of known Northern Buller's Albatross material to estimate the degree of differentiation between the two Buller's Albatross taxa. A previous attempt to use genetic markers to differentiate between Northern and Southern Buller's Albatross did not have access to a very large sample of Northern Buller's Albatross (van Bekkum 2004). DNA sequence diversity at the CRII locus is similar to the levels of nucleotide and haplotype diversity reported for Domain I of the control region sampled from other Procellariiformes species (Alderman *et al.* 2005; Burg & Croxall 2001; Lawrence *et al.* 2008). This is not surprising as Abbott *et al.* (2005) noted that both CRI and CRII were largely evolving in concert, despite a short independently evolving section within each. It believed that the F2 fragment of CRII is a neutral region (Abbott *et al.* 2005), and is not linked with other mitochondrial genes under selection. Neutral DNA sequences are able to accumulate mutations and require very significant and diversifying selection to depart from assumptions of neutrality (Le Corre & Kremer 2003).

The high level of variability found at CRII is consistent with its characterisation as a non-coding region. Therefore, the results of neutrality tests conducted during this study have been interpreted as genetic variation that has been primarily influenced by demographic processes.

Haplotype and nucleotide diversity levels within Northern Buller's Albatross were slightly higher than within Southern Buller's Albatross (Table 2.2). The lower levels of diversity and the significantly negative neutrality values within Southern Buller's Albatross may be indicative of a few scenarios: 1) Southern Buller's Albatross may have been founded from a range expansion of Buller's albatrosses southward; 2) Southern Buller's Albatross populations are undergoing an expansion, independent of range expansion by Buller's Albatross populations; or 3) Southern Buller's Albatross experienced a recent population bottleneck (Fu 1997; Tajima 1989).

The shape of the haplotype network is a starburst-like pattern, which is consistent with the suggestion of a recent population expansion (Slatkin & Hudson 1991). When population size increases, a larger number of new mutations can persist. This causes the overall impact of genetic drift on a population to decrease, allowing for the persistence of more haplotypes. During the initial expansion of a population, haplotypes occur at a relatively low frequency and are only separated by a few mutational steps (*see* Figure 2.4). In contrast, the pattern of a historically stable population is evident in the pattern of Northern Buller's Albatross haplotypes. In this case the haplotype network is made up of many distinct haplotypes that occur at a relatively low to moderate frequency and are separated from each other by many mutational steps (Slatkin & Hudson 1991). However, the haplotype network does not distinguish between the possibility that Southern Buller's Albatross arose from a demographic expansion of Buller's albatrosses southward or if the population growth is independent of a range expansion. The contrasting patterns between Northern and Southern Buller's Albatross support the suggestion that the two groups have been separated for long enough to experience different demographic histories.

Genetic Structure

Sequence divergence between Northern and Southern Buller's Albatross haplotypes varied from 1.4 – 6.8%. As a result of the exceptionally wide range of variation within each group, there was no clear distinction between the differences within and between the two taxa (Figure 2.2). Depending on which fixation measurement was used, there is either a low (F_{ST}) or high (Φ_{ST}) degree of regional differentiation between Northern and Southern Buller's Albatross. The exceptionally high levels of sequence diversity within Buller's Albatross may have confounded AMOVA and pairwise F_{ST} measures of divergence between the two regions.

Northern and Southern Buller's Albatross exhibit similar levels of haplotype diversity, and have an excess of rare haplotypes as revealed by the Fu's F_S values. Pairwise F_{ST} calculations consider all distinct haplotypes equidistant from one another and assess the relative frequency of haplotypes within a defined group. As a result, pairwise F_{ST} values are indicative of differences in diversity patterns within the two groups, rather than a true measure of differentiation. In the case of Buller's Albatross, the pairwise F_{ST} value between the two regions indicate that Northern and Southern Buller's albatrosses have a small, but significant, difference in the relative frequency of haplotypes. This is particularly notable, as the two taxa do not share haplotypes. Fixation indices that incorporate distance among haplotypes (Φ_{ST}–statistics) can provide more informative results, because they incorporate an absolute measure of genetic distance between populations and regions (Excoffier *et al.* 1992). In contrast to regional pairwise F_{ST}, regional pairwise Φ_{ST} values showed high levels of differentiation between Northern and Southern Buller's albatrosses (regional pairwise Φ_{ST} = 0.62). Pairwise Φ_{ST} values are consistently high, and significant, between colonies sampled in different regions. While pairwise Φ_{ST} between colonies within a region are insignificant (Table 2.3). However, these high values may be misleading. Since the family of F-statistics were developed for biallelic nuclear genes. The application of this class of statistics to mitochondrial data may result in inflated values. This is because the mitochondrial genome is haploid and

maternally inherited, reducing the effective population size to ¼ that of a nuclear locus (Avise 2000; Slatkin 1987). Nevertheless, these regional and colony level comparisons indicate Northern and Southern Albatross are genetically differentiated.

Both the AMOVA calculated using genetic distance (Table 2.4), and the AMOVA calculated with haplotype frequency (*see* Appendix II) showed that differentiation between regions, and between colonies within regions, was insignificant. These AMOVA results are partially consistent with pairwise colony comparisons (Table 2.3), which indicate that colonies within a region are not differentiated. The lack of genetic structure among colonies within a region in this study is consistent with a previous microsatellite DNA study of Southern Buller's Albatross (van Bekkum *et al.* 2006).

Interestingly, both AMOVA indicated that variation was significant at the colony level *(*$\Phi_{ST} = 0.62$, $p < 0.00001$; $F_{ST} = 0.03$, $p < 0.05$). However, the variance at the colony level is roughly equal to that between regions (Table 2.4), which have potentially skewed the null distribution. As a consequence, the AMOVA could not distinguish between variation at the colony and regional level. This leaves differentiation between regions as insignificant, despite the two regions not sharing haplotypes. This was a contrast to findings of pairwise differences where colonies between regions resulted in very high levels of differentiation (i.e. Motuhara vs. Solander Island; Table 2.3).

Regional differentiation is further supported by the isolation of haplogroups to either the Northern or Southern groups with Bayesian Analysis of Population Structure (BAPS). Despite the overlap in inter- and intra-group pairwise differences between Northern and Southern Buller's Albatross (Figure 2.2), the two taxa formed distinct groupings when graphed as a haplotype network (Figure 2.4). The ambiguous haplotype from Motuhara sits between the two groups, and is identified by BAPS as more closely related to the Southern haplogroup. This haplotype may be a relic of incomplete lineage sorting between the two geographically separate subspecies. Given that no bycatch samples shared the ambiguous Motuhara haplotype, and the high level of variation observed within the Northern population, the haplotype is likely

rare within Northern Buller's Albatross. However, increasing the sample sizes would be helpful to provide better support for this conclusion. Furthermore, the addition of nuclear DNA loci would help determine the bi-parental estimates of genetic connectivity between populations and facilitate testing for the effects of recent population bottlenecks using a range of independent loci (Neigel 1997; Waples & Gaggiotti 2006).

Natal philopatry may play an important role in the process of increasing genetic differentiation between the Northern and Southern groups. Birds banded at Southern Buller's Albatross colonies are known to have high site fidelity and observational data show that they return to the same nests year after year (Sagar *et al.* 1998). Asynchronous breeding seasons of the Northern and Southern groups could be a strong mechanism to limit gene flow. Northern Buller's Albatross return to colonies and begin breeding two to three months prior to Southern Buller's Albatross (Robertson & Sawyer 1994; Robertson *in prep.*). Since incubation typically lasts 68 – 72 days (Sagar & Warham 1998), by the time Southern Buller's Albatross are starting to lay northern eggs have already begun hatching (*see* Appendix II). Finally, Albatross invest heavily in parental care for a single chick. As a result, it is unlikely either parent would go south to mate again.

Friesen *et al.* (2007) noted a strong correlation between significant levels of genetic differentiation and population-specific, non-breeding areas or seasons in a number of seabird taxa. While the asynchronous breeding season between the two groups is well known (Fraser *et al.* 2008; Robertson & Sawyer 1994; Sagar & Warham 1998), it is unknown if there is a geographical difference in nonbreeding areas between Northern and Southern Buller's albatrosses. The at-sea distribution of Southern Buller's Albatross has been well documented and is recorded in the Tracking Ocean Wanderers Project (BirdLife International 2004). This database is a collection of studies which have implemented platform terminal transmitters (PTT's) and GPS tracking data to represent the distribution of Buller's Albatross sp. as a whole (*see* Figure 1.2; Broekhuizen *et al.* 2003; Deppe 2008; Sagar & Weimerskirch 1996; Stahl & Sagar 2000a, b). However, data for the at-sea distribution of Northern

Buller's Albatross is limited to two tracking records collected between the 10th and 15th of November, 2008 (Deppe 2008) and cannot be used to represent the distribution of the Northern taxon. The genetic differentiation observed in this study and the reports of asynchronous breeding seasons between regions, suggest that the two taxa might have distinct distributions during the breeding and nonbreeding season. The behaviour of individuals from different populations at sea may have significant implications for the types of threats they encounter.

Assignment of Bycatch

The idea that genetic differentiation could be used to assign bycaught procellariiform individuals to their population of origin was first proposed by (Edwards *et al.* 2001b) and has since been applied to a number of seabird species. For example, Burg (2007) and Militao *et al.* (2014) utilised frequency based assignment methods alongside other metrics, such as nuclear markers, morphology, or stable isotope analysis to differentiate recently diverged species and subspecies. More recently, Inoue *et al.* (2015) applied an assay of RFLP's. However this method was unable to resolve among subspecies.

In the present study, the performance of a 221 bp fragment of CRII in assigning the provenance of individuals was assessed. This was done primarily by characterising genetic variation within and between Northern and Southern Buller's albatrosses. The results showed that the two taxa are highly diverse and as a result, the test of structure between the two groups is difficult to assess. However, two exclusively Northern haplogroups and one Southern haplogroup were identified using BAPS and visualised on a haplotype network (Figure 2.3, Figure 2.4). All 97 bycatch samples could be unambiguously assigned to one of the three major haplogroups. Nineteen were identified as Northern Buller's Albatross and the remaining 78 as Southern Buller's Albatross.

It is clear that Northern Buller's Albatross are represented in New Zealand fisheries bycatch. As Buller's Albatross ssp. bycatch is accumulated and assigned, the relative proportions of the Northern and Southern taxa in New Zealand's commercial fisheries bycatch will become clearer. The presence

of 20% Northern Buller's Albatross in this relatively small bycatch study demonstrates the need for increased monitoring of Northern Buller's Albatross colonies and the need to produce better estimates of incidental mortality. Expanding the Northern and Southern Buller's Albatross genetic reference dataset is integral to improving the range of reference DNA sequences. A better estimate of the genetic diversity within each subspecies group will increase certainty of assignment to a population of origin. While assignment to taxon is successful, both taxa were under sampled and did not adequately represent the diversity found within each region. This study only assessed roughly 16% of Buller's Albatrosses caught within New Zealand's EEZ over the last 20 Years (*see* Appendix III). An expansion of the sampling across years would result in a more comprehensive assessment on the overall proportion of Buller's Albatross ssp. in the seabird bycatch. This information could be used to indirectly aid population studies by assessing the role of seasonality and identifying overlaps between commercial fisheries and the at sea distribution of Northern and Southern Buller's Albatross.

This study reported a reliable DNA-based method for identifying Northern and Southern Buller's Albatross. It can be used to assign individuals to their provenance even if a specimen has been presented in poor physical condition. The ability to accurately identify Northern Buller's Albatross individuals is an important new capability, which will enable the impact of incidental mortality on the Northern Buller's Albatross to be properly assessed. However, there are still gaps in the estimates of population size and vital ecological statistics for the Northern taxon. A more extensive study of Northern Buller's Albatross colonies may benefit a number of other seabirds that share breeding grounds, such as the Northern Royal Albatross (*Diomedea sanfordi*) and Northern Giant Petrel (*Macronectes halli*) (Robertson & Sawyer 1994). Finally, the method presented in this thesis research could be expanded to include to a number of other threatened birds to better assess the impacts of fisheries related mortality.

CHAPTER 3 GENE FLOW AND GENETIC CONNECTIVITY IN NORTHERN AND SOUTHERN BULLER'S ALBATROSS (*THALASSARCHE BULLERI* SSP.)

Abstract

Seabirds are known for their ability to navigate great distances throughout the world's oceans. Despite this dispersal capability, many taxa exhibit high fidelity to natal colonies. Site fidelity is believed to limit migration among breeding groups. The Buller's Albatross species complex is composed of two asynchronously breeding groups, the Northern Buller's Albatross (*Thalassarche bulleri platei*) and the Southern Buller's Albatross (*Thalassarche bulleri bulleri*). While a previous study found no evidence for genetic differentiation among Southern Buller's Albatross colonies, to date there have been no investigations into the genetic or demographic connectivity between Buller's Albatross ssp. The aim of this study was to test for genetic differentiation between Northern and Southern Buller's Albatross. Genotyping-by-Sequencing (GBS) was used to assess levels of gene flow and genome wide divergence using 14 *T. b. platei* and 35 *T. b. bulleri* samples. Three filtering protocols were applied to 54,061 single nucleotide polymorphisms (SNPs) within 26,319 putative loci. Nucleotide diversity and heterozygosity estimates for all sites were similar across taxa and filtering protocols ($\pi \approx 0.001 - 0.004$; $H_O \approx 0.002 - 0.003$). Estimates of genome-wide differentiation between taxa were low, but increased slightly as more restrictive filtering protocols were applied ($F_{ST} \approx 0.018 - 0.038$). Pairwise F_{ST} values utilising only variable loci indicated low, but significant levels of differentiation between taxa (pairwise $F_{ST} = 0.111$) and between colonies within a taxon (pairwise $F_{ST} = 0.018$). Under the most restrictive filtering parameter, tests for F_{ST} outliers suggested that some loci are under directional selection. However, these loci should be mapped back to a reference genome to distinguish between selection and divergence at neutral loci. A STRUCTURE analysis consistently showed highly differentiated clusters corresponding to Northern and Southern taxa. The

findings of this study indicate that an asynchronous breeding season has contributed to limited gene flow between breeding groups of Buller's Albatross.

Introduction

Seabirds are a paradox for population genetics. Their high dispersal capability means the physical distance between colonies may not pose a significant barrier to gene flow (Friesen 2015; Friesen *et al.* 2007), yet albatross species exhibit both range-wide connectivity and colony differentiation over short distances (*see* Munro & Burg 2017 *for review*). Range-wide genetic connectivity has been reported for the Wandering (*Diomedea exulans*), Grey-headed (*Thalassarche chrysostoma*), and Black-browed albatrosses (*T. melanophris*) (Burg & Croxall 2001, 2004). However, connectivity for other species appears to be restricted over relatively short distances, such as in Antipodean (*D. antipodensis*) and Shy (*T. cauta*) albatrosses (*see* Munro & Burg 2017 *for review*). The oceans in the southern hemisphere are relatively more contiguous than those in the northern hemisphere. As a result, geographical and physical barriers to dispersal are superseded by behavioural factors in the structuring of seabird populations (Munro & Burg 2017). Many seabirds are highly philopatric (Coulson 2002; Silva *et al.* 2015), which can potentially limit gene flow and dispersal between colonies (Lawrence *et al.* 2014). However, genetic differentiation has also been associated with morphological differences (Bost *et al.* 1992; Jouventin *et al.* 2006), variation in at-sea distribution (Burg & Croxall 2001; Friesen 2015; Friesen *et al.* 2007), and breeding phenology (Brown *et al.* 2015; Henry & Day 2005). The Northern Buller's Albatross (*Thalassarche bulleri platei*) and the Southern Buller's Albatross (*T. b. bulleri*) present a challenge for inferring population structure due to uncertainty regarding morphological differentiation and in the at-sea distribution of Northern Buller's albatrosses as well as a highly divergent breeding phenology.

Currently, identifying these taxa with morphological characteristics may be problematic as there is some evidence to suggest that head and brow plumage can be used to identify the subspecies (*see* del Hoyo & Collar 2014; Robertson 1985), but this trait has not been critically assessed. The lack of clear

diagnostic characteristics suggests that there may be very little genetic difference between the two groups. A previous microsatellite study found that although individuals are philopatric (Sagar *et al.* 1998), this did not appear to have resulted in genetic structure among Southern Buller's Albatross colonies (van Bekkum *et al.* 2006). It is currently unknown whether the two taxa have at-sea distributions that vary temporally or geographically. While the at-sea distribution of Southern Buller's albatrosses is well recorded (BirdLife International 2004), there have been no significant investigation into the at-sea distribution of Northern Buller's albatrosses.

Asynchronous breeding periods have been linked to genetic differentiation in species, such as the Northern (*Macronectes halli*) and the Southern (*M. giganteus*) Giant Petrel (Brown *et al.* 2015). Some Northern and Southern Giant petrel breeding sites are sympatric (Bourne & Warham 1966), yet hybridisation rates between the two taxa are relatively low (Brown *et al.* 2015). This low rate of hybridisation has been attributed to their asynchronous breeding phenology. A study conducted by Brown *et al.* (2015) investigated the hybridisation of Northern and Southern Giant Petrels on Bird Island, South Georgia. It was observed that Northern Giant Petrel lay dates typically ranged from mid-September to mid-October. In contrast, Southern Giant Petrels lay dates extended from late October until late November. Hybrid Giant Petrels generally had lay dates that were late for Northern Giant Petrels and always prior to Southern Giant Petrels (Brown *et al.* 2015). This potentially impacted fitness, as hybrid breeding success rates were lower than those of conspecific pairs (Brown *et al.* 2015). Breeding phenology may serve as a pre-zygotic barrier to hybridisation and gene flow when asynchronous breeding occurs. In the case of Buller's Albatross ssp., Northern Buller's albatrosses return to breeding colonies around the Chatham and Three Kings Islands three months prior to the return of Southern Buller's albatrosses to their breeding colonies (Robertson *in press; see* Appendix III). It is probable that asynchronous breeding reduces the opportunities for inter-mixing between groups. This has led to the suggestion that the two taxa may warrant full species recognition (Robertson & Nunn 1998). Yet, to date, there have been no investigations into

the degree of genetic or demographic connectivity between Northern and Southern Buller's Albatross ssp.

Analysis of the mitochondrial control region, Domain II, showed that there are significant genetic differences between the two groups and that the southern group likely arose from a range expansion southward (*see* Chapter 2). However, it remains difficult to determine whether this population subdivision is the result of high female philopatry or if the two groups were historically separated and have come into secondary contact. Bi-parentally inherited nuclear markers would provide a useful comparison to the maternally inherited mtDNA and help resolve the contemporary patterns of gene flow.

Parallel sequencing techniques enable genome-wide variation to be assessed, which vastly increases the statistical power of genetic analyses. This allows fine-scale patterns of gene flow between, or within, populations to be resolved. Genotyping by Sequencing (GBS) is a relatively flexible, low cost method for genome-wide Single-Nucleotide Polymorphism (SNP) discovery using a population-level sample of individuals. Some benefits of GBS include the ability to simultaneously genotype multiple samples and to sequence a range of species without much modification (Elshire *et al.* 2011). Genome-wide SNP data can potentially be used to detect areas of the genome under selection, as well as the more general processes of genetic drift and gene flow (Davey *et al.* 2011). The aim of the research in this chapter was to collect genome-wide SNP data using GBS for Northern and Southern Buller's Albatross samples and to determine the relative degree of gene flow between colonies and taxa. The results of this study may provide insights into the potential role of asynchronous breeding seasons in maintaining genetic differentiation between Buller's Albatross taxa.

Methods

Sampling and DNA Extraction

DNA was extracted from 96 blood samples of Buller's Albatross ssp. from two northern colonies (23 = Motuhara; 6 = Rangitatahi), and two southern colonies (43 = North East Island; 24 = Solander Island). Blood samples were

stored in ethanol and kept at -4°C prior to DNA extraction. Blood and tissue samples were digested in extraction buffer (10 mM Tris pH 8.0, 50 mM NaCl, 10 mM EDTA, and 0.2% SDS) with 0.5 µg/µL proteinase-K. The DNA was extracted using phenol and chloroform solutions as per Sambrook *et al.* (1989). Total DNA was precipitated using ethanol, dried, and re-suspended in 30 µL TE buffer (10mM Tris. pH 8.0, 1mM EDTA). The purified DNA was aliquoted into a 96-well plate, covered and stored at 4°C prior to analysis.

GBS data collection: restriction enzyme digestion and Illumina Sequencing (conducted by AgResearch in Mosgiel, New Zealand)

While genome size estimates are unavailable for Procellariiformes, genome size estimates for the class of Aves range from 890 Mbp to 2,113 Mbp, (Gregory 2017, www.genomesize.com). To ensure adequate digestion of the genome, an assay of *Ape*KI, *Ape*KI/*Msp*I, *Pst*l, and *Pst*l/*Msp*l was assessed using a high quality *T. bulleri* ssp. DNA extraction. Restriction enzyme reactions were standardised to volumes of 1.5 µL and 100 ng of template DNA. Digestions using *Ape*KI were incubated at 75°C for 2 hours. Digestions with *Pst*l and/or *Msp*l were initially incubated at 37°C for 2 hours, followed by 30 minutes at 65°C. Restriction enzyme fragments were visualized using gel electrophoresis. Fragment size and concentration were estimated against a standardized GeneRuler 1 kb DNA ladder (*see* Appendix IV). Digestion with *Ape*KI yielded fragments between 700 – 3,000 bp, *Ape*KI/*Msp*I yielded 1,000 – 5,000 bp fragments, *Pst*l yielded 2,000 – 7,000 bp fragments, and *Pst*l/*Msp*l yielded 1,000 – 4,000 bp fragments. The restriction enzyme *Pst*l was chosen to increase the likelihood that samples would have a high depth of DNA sequence reads. After digestion, restriction fragments were tagged with DNA barcode sequences that identified individuals prior to pooling for PCR amplification (Elshire *et al.* 2011). The *Pst*l 95-plex GBS library was purified and an Agilent Bioanalyzer was used to evaluate the fragment sizes and adapter dimers (AgResearch, Mosgiel New Zealand). The library was sequenced using an Illumina sequencing platform (AgResearch, Mosgiel New Zealand).

GBS data preparation: de novo read alignment and SNP filtering (conducted by Kate McKenzie)

The pre-processing and genotyping protocols implemented for this data set have been modified from Dierickx *et al.* (2015) and were conducted by McKenzie (2016). The STACKS software pipeline was used to build putative loci *de novo*. A 'stack' is the alignment and grouping of fragment reads. The 'depth' of a stack is the number of similar overlapping sequence reads that can be assembled for a short sequence region.

Because there are no whole-genome sequences available for any seabird species to use as a reference, the STACKS v1.37 (Catchen *et al.* 2011; Catchen *et al.* 2013) *de novo* variant calling pipeline was used for SNP calling. In the *de novo* pipeline, each stack of sequence reads was considered a putative locus. Without a reference genome for mapping loci, the distance among, and relative positioning of, sequence reads is unknown. In the construction of a *de novo* alignment for Buller's Albatross ssp. reads were demultiplexed and examined independently for each sample. To account for low depth of coverage, cores were assembled and marked as *primary* if the *minimum-stack-depth* parameter (–m) was greater than 3 (*see* Catchen *et al.* 2013). It is important to note that the stack depth described here is the initial assemblage of cores, where similar reads are grouped together, and it is not the final depth of the assembled stack that can include multiple alleles.

The sequence similarity of cores was compared to determine levels of heterozygosity. The minimum read depth to call heterozygous loci is *populations* –m = 8 (*see* Catchen *et al.* 2013). This is because calling a heterozygous locus requires a reasonable depth of reads to distinguish an allele from sequencing error. In datasets with low depth, there is a bias away from heterozygous loci. While the default value for the *populations* minimum stack depth (*populations* –m = 3) was used for assembling data sets, the effect of low sample read depth on heterozygosity estimates was investigated by varying *populations* –m from –m = 0 to –m = 20. Values were graphed to assess the bias against heterozygous sites in this data set.

Cores with *distances-within-samples* (M) less than 4 were merged following the approach of Catchen *et al.* (2013). Because stacks composed of 3 or more cores are likely the result of over-merging of repetitive sequences or PCR error, deleveraging was used to split these stacks into subsets. If the depth of a given stack remained high after this process, it was discarded. The remaining secondary stack cores were included only if the mismatch was less than the default value of five. For the aggregation of loci, a reference catalogue was generated to ensure a consistent reference for loci. This catalogue was used to correct genotype and haplotype calls in individual samples. For a locus to be merged with similar catalogue loci, it must not exceed the defined *distance-between-samples* (n). After assessing the effects of varying n, a value of 1 was determined to be most appropriate.

To ensure that the data sets for the northern and southern taxa were comparable, putative loci that occurred only in the northern or southern taxon were removed. To investigate the effects of the three filtering procedures on estimates of genetic diversity and differentiation, separate data sets (A, B, & C) were made for each set of filtering criteria. Data set A required loci to be present within at least 50% of individuals of each taxon. Data set B required loci to be present within at least 50% of individuals of each taxon and only used the first SNP on each putative locus. Data set C required loci to be present within at least 50% of individuals of each taxon, only used the first SNP on each putative locus, and excluded loci with heterozygosity values >0.75 and minor allele frequencies (MAF) <0.05. After *de novo* alignment and SNP filtering, a sample coverage threshold of 80% was enforced to ensure individuals were comparable and to ensure confidence in genotyping. If a sample did not reach this threshold it was removed from further analyses.

GBS data analysis: population differentiation and gene flow

The software package Kinship-based INference for Genome-wide association study (KING) was used to assess relatedness among individuals to ensure only unrelated individuals were included in the data set (Manichaikul *et al.* 2010). Overall F_{ST}, nucleotide diversity (π), heterozygosity, and per site estimates of F_{ST} and π were estimated using STACKS. The *adegenet,* (Jombart

2008; Jombart & Ahmed 2011) and *hierfstat* (Goudet & Jombart 2015) packages in R v3.2.3 (R Core Team 2015) using Rstudio v1.0.136 (Rstudio Team 2016) were used to calculate pairwise F_{ST} between taxa and between individual colonies as per Weir and Cockerham (1984). Heterozygosity estimates per site, and Hardy-Weinberg Equilibrium (HWE) (Hardy 1908; Weinberg 1908) were also estimated with the package *pegas* (Paradis 2010). Confidence intervals for pairwise F_{ST} and HWE estimates were generated with 1,000 replicates. While per site heterozygosity was compared across data sets A – C, only data set C was used to calculate pairwise F_{ST} and HWE because it was the least likely to be confounded by sequencing or pipeline filtering errors.

BayeScan v2.1 (Foll & Gaggiotti 2008) was used to test for F_{ST} outliers in all three datasets (A, B & C). Outlier tests were run under the default settings. BayeScan results were visualised in RStudio with the BayeScan source code and the *graphics* package (R Core Team 2015). STRUCTURE v2.3.3 (Pritchard *et al.* 2000) was used to assess the degree of admixture between Northern and Southern Buller's Albatross and between the two Southern Buller's Albatross colonies (North East Island and Solander Island). Loci from data set C were used to determine the number of clusters (K) and assign individuals to clusters, as this data set was least likely to be biased by genetic linkage. STRUCTURE was run in replicates of 3 with a burn-in of 100,000 steps and 300,000 Markov chain Monte Carlo samples for each value of K from K = 1 to K = 6. The Evanno method (ΔK; Evanno *et al.* 2005) was used to choose the optimum value of K as implemented in STRUCTURE HARVESTER v0.6.94 (Earl & vonHoldt 2012). Replicate result files were combined using CLUMPP v1.1.2 (Jakobsson & Rosenberg 2007) and visualised with *distruct* v1.1 (Rosenberg 2003). To further investigate patterns of structure among colonies of Buller's Albatross ssp., a principal coordinate analysis was conducted in Rstudio with the *hierfstat* package.

Results

Genotyping by sequencing SNPs as per Kate McKenzie

The initial quality assurance checks identified two pairs of samples with relatedness values > 0.9. These samples were dropped from the data set. An additional 39 samples were excluded because of low quality template DNA, poor quality calls, and low retained read counts. The northern breeding colony, Rangitatahi (n = 3), was dropped from analyses, despite high confidence reads, as > 90% of the loci were missing for each individual. A total of 49 samples from 3 breeding colonies were retained, 14 were representative of Northern Buller's Albatross (Motuhara), and the remaining 35 were representative of Southern Buller's Albatross (23 = North East Island; 12 = Solander Island). Retained samples had a mean sample stack depth of –*m* = 8.

The Illumina sequencing yielded 281,507,853 first-end reads. After the file was cleaned and demultiplexed, 10,353,525 reads were discarded due to errors in the barcode sequence or the lack of an intact restriction enzyme cut site. An overrepresented sequence from the 3' adaptor was identified (FastQC report, AgResearch 2016) in many reads due to fragments being shorter than the standard read length (101 bp). To increase read retention, the reads were truncated at nucleotide position 78; this removed the adaptor content from the 3' end. An additional 126,653,299 reads were discarded due to low sequence quality or remaining adaptor content. The *de novo* alignment process utilized 9,648,376 reads to assemble 7,517,479 putative loci. After loci aggregation, and genotyping quality checks 257,789 putative loci containing 233,920 SNPs were left for SNP filtering. Initially, as the minimum stack depth (*populations* –m) was increased, observed heterozygosity increased. However, observed heterozygosity plateaued around –m = 10 (Figure 3.1). After locus filtering protocols, data set A comprised of 26,319 putative loci containing of 54,061 SNPs remained. Data set B was composed of 26,287 SNPs of high confidence in as many putative loci. Finally, data set C was left with 17,235 SNPs (Table 3.2, Table 3.1)

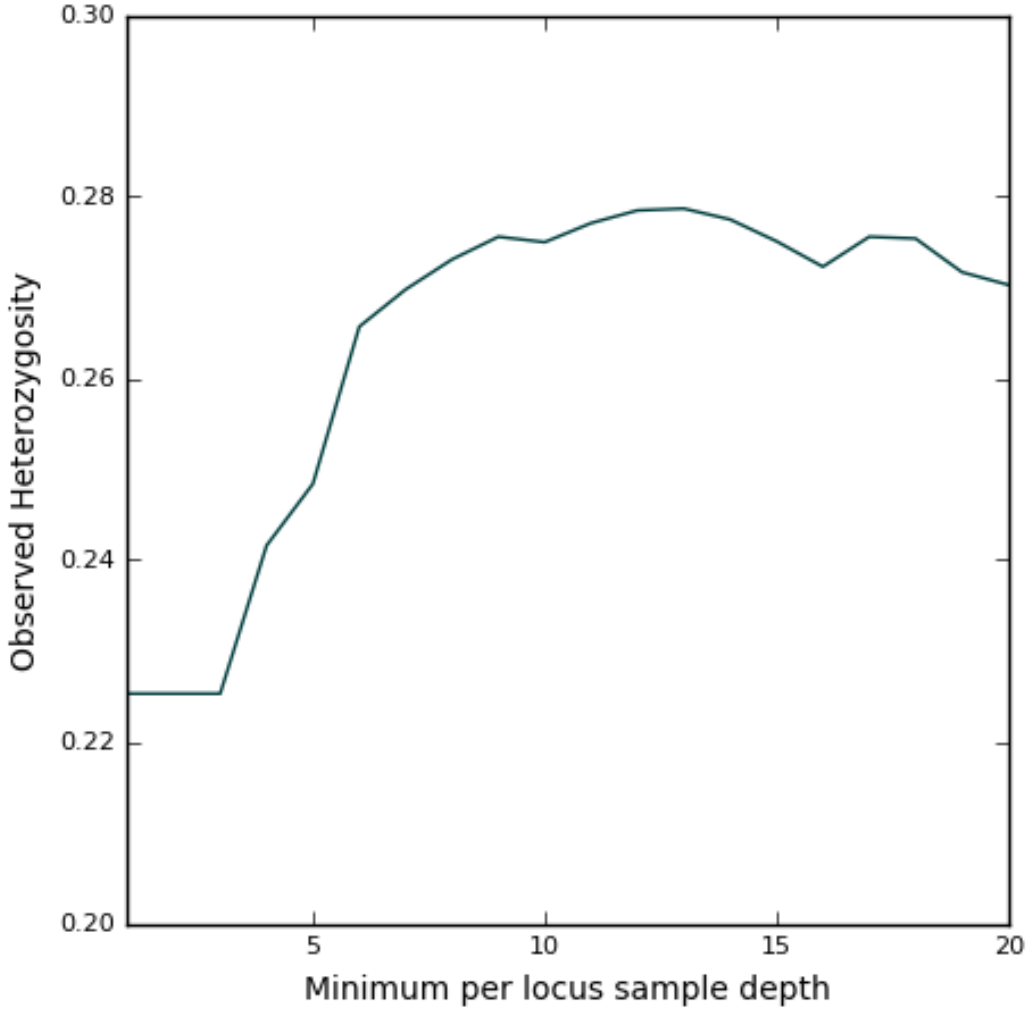

Figure 3.1 Observed heterozygosity as a function of minimum per locus sample depth (-m)
To investigate the effect of pipeline settings on genetic diversity estimates, minimum per locus sample depth was varied from –m = 0 to –m = 20.

Table 3.2 Summary of data sets A – C and their corresponding single nucleotide polymorphism (SNP) filtering protocols

Three SNP filtering protocols were used to assess the effect of SNP filtering on differentiation and genetic diversity estimates for Buller's Albatross ssp. If loci met the initial presence threshold (A), the number of loci per putative locus (B), observed heterozygosity and minor allele frequency thresholds (C) were used to apply increasingly restrictive requirements for SNP inclusion.

Data set	Presence requirement for locus inclusion	SNP filtering	Number of SNPs
A	Present in at least 50% individuals in each region	None	54,061
B		1st SNP / locus	26,287
C		1st SNP / locus , heterozygosity < 0.75, and MAF > 0.05	17,235

Table 3.1 Summary of GBS run information and genetic diversity indices estimated with three filtering protocols as calculated in STACKS v1.37

Summary of Genotyping by Sequencing data applied to three distinct SNP filtering protocols A, B and C (*see* Table 3.2), as well as overall heterozygosity and overall nucleotide diversity (π) estimates for each. Sites are defined as individual positions within sequences, each 78 bp read was utilised as a putative loci for downstream analysis.

	Northern Buller's Albatross (*T. b. platei*)			Southern Buller's Albatross (*T. b. bulleri*)		
All Positions	**A**	**B**	**C**	**A**	**B**	**C**
Number of putative Loci	26,319	26, 285	17,235	26,319	26, 285	17,235
Total number of sites	2,052,770	2,052,848	1,345,639	2,052,770	2,052,848	1,345,639
Observed heterozygosity	0.0032	0.0020	0.0031	0.0026	0.0016	0.0029
Expected heterozygosity	0.0036	0.0022	0.0036	0.0033	0.0020	0.0037
Observed homozygosity	0.9968	0.9980	0.9969	0.9974	0.9984	0.9971
Expected homozygosity	0.9964	0.9978	0.9964	0.9967	0.9980	0.9963
Nucleotide diversity (Overall)	0.0038	0.0023	0.0038	0.0023	0.0013	0.0026
Variant Positions Only						
Number of SNPs	54,059	26,285	17,235	54,059	26,285	17,235
Number of private alleles	17,705	6,877	452	17,885	6,877	2,000
Observed heterozygosity	0.1221	0.1541	0.2402	0.0990	0.1273	0.2253
Expected heterozygosity	0.1376	0.1731	0.2821	0.1237	0.1583	0.2889
Observed homozygosity	0.8779	0.8459	0.7598	0.9010	0.8727	0.7747
Expected homozygosity	0.8624	0.8269	0.7179	0.8763	0.8417	0.7111
Nucleotide diversity π (Overall)	0.0607	0.0735	0.1451	0.0859	0.1036	0.2052

Genetic diversity and gene flow

All individuals used in this study did not show evidence of being close relatives. Both estimates of overall and per site nucleotide diversity (π) were similar across data sets (Table 3.1, Table 3.2). However, per site F_{ST} was similar only between data sets A and B (Figure 3.2). The distributions changed as heterozygosity and MAF were limited (Data set C; Figure 3.2). Overall differentiation between taxa was small across data sets, but showed a slight increase as filtering protocols became more restrictive: overall F_{ST} = 0.018 (A), 0.024 (B) and 0.038 (C). Comparisons of overall observed and expected heterozygosity were similar across data sets (Table 3.1), yet heterozygosity estimates for variant sites increased as SNP filtering protocols became increasingly restrictive. Distributions of per locus π, and per locus F_{ST} were similar for data sets A & B. However, after the MAF and heterozygosity limits were applied in data set C, the lower bound of per locus π, and per locus F_{ST}

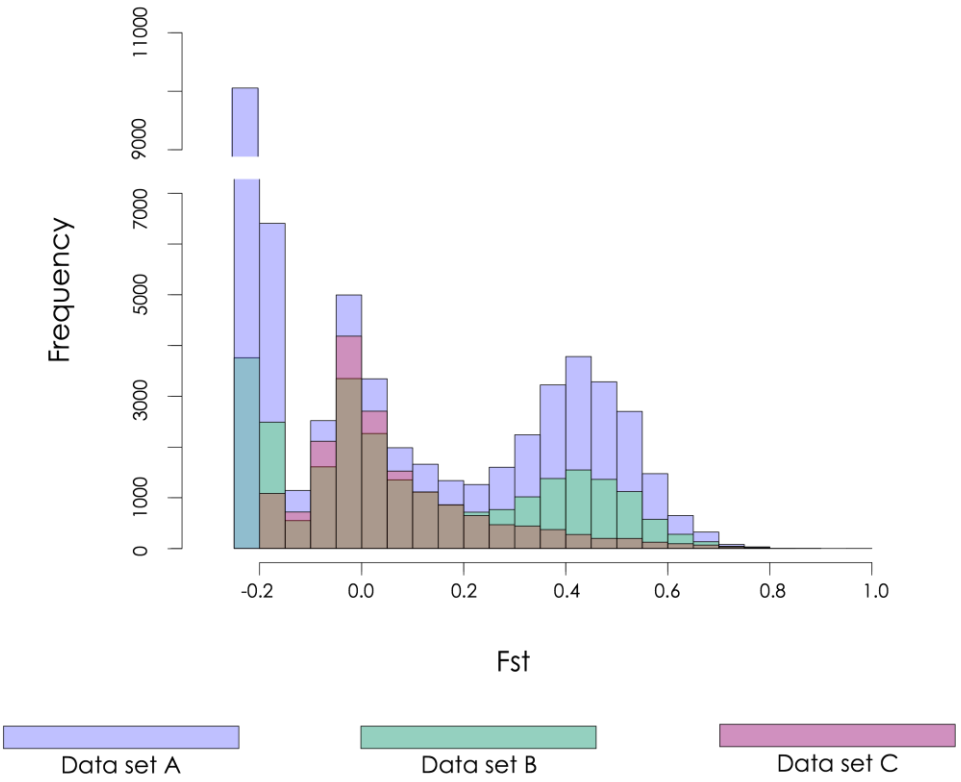

Figure 3.2 Estimates of per locus nucleotide diversity, and F_{ST}
Distributions of per locus F_{ST} values calculated with data sets A, B & C for both Northern and Southern Buller's Albatross. Brown colour denotes overlap in all three data sets.

Table 3.3 Pairwise F_{ST} comparisons between colonies of Buller's Albatross ssp.

Pairwise comparison matrix of F_{ST} values among Buller's Albatross colonies as per Weir and Cockerham (1984). Values below the diagonal are pairwise F_{ST} estimates, while values above the diagonal are the lower bound of confidence intervals obtained with 1,000 replicates. Motuhara is a Northern Buller's Albatross colony (*T. b. platei*), while Solander Island and North East Island are Southern Buller's Albatross colonies (*T. b. bulleri*).

Colony	Motuhara	Solander Island	North East Island
Motuhara	-	0.119	0.108
Solander Island	0.122	-	0.016
North East Island	0.111	0.018	-

increased. In addition, the upper bound for per locus F_{ST} no longer demonstrated a second peak (Figure 3.2; Appendix V).

Pairwise F_{ST} values estimated with data set C indicated moderate levels of differentiation between Northern and Southern Buller's albatrosses (pairwise $F_{ST} = 0.111$). Comparisons between the three Buller's Albatross ssp. colonies were significant, with pairwise F_{ST} values between northern and southern colonies an order of magnitude greater than the comparison between southern colonies (Table 3.3). Estimates of HWE were not calculated for data set A as there were multiple SNPs per putative loci, this proved difficult to calculate and to properly take all SNPs per putative locus into consideration. Within data set B, 4,486 out of 26,285 loci were not in HWE, and of the 17,235 loci included in data set C, 5,739 were not in HWE.

There were no F_{ST} outliers in data sets A and B. However, there were 16 outliers in Data set C (Figure 3.3). Because STRUCTURE can be sensitive to linked loci, and the results of the other datasets are qualitatively similar, only data set C was used to conduct STRUCTURE analyses. The log-likelihood of the model was similar when K = 2, 3, and 5 were used, with K = 2 being favoured by ΔK. All three models divided Northern and Southern Buller's Albatrosses into two distinct groups. Admixture between Northern and Southern Buller's albatrosses was not evident (Figure 3.4). Population structure between the two Southern Buller's Albatross colonies, Solander Island and North East Island was confirmed in a separate STRUCTURE analysis. Two clusters were identified among southern colonies (ΔK = 2), and while admixture patterns

were not as clear as comparisons among Buller's Albatross ssp., each cluster largely corresponded to one of the two southern colonies (Appendix VI). This taxa level population structure and colony level sub-structure was further supported by a principal coordinate analysis (PCA; Figure 3.5). Northern and Southern Buller's Albatross clustered into two distinct groups with Southern Buller's Albatross colonies clustering into a generally large group. While individuals representative of southern colonies were closely grouped, there was little overlap between the two (Figure 3.5).

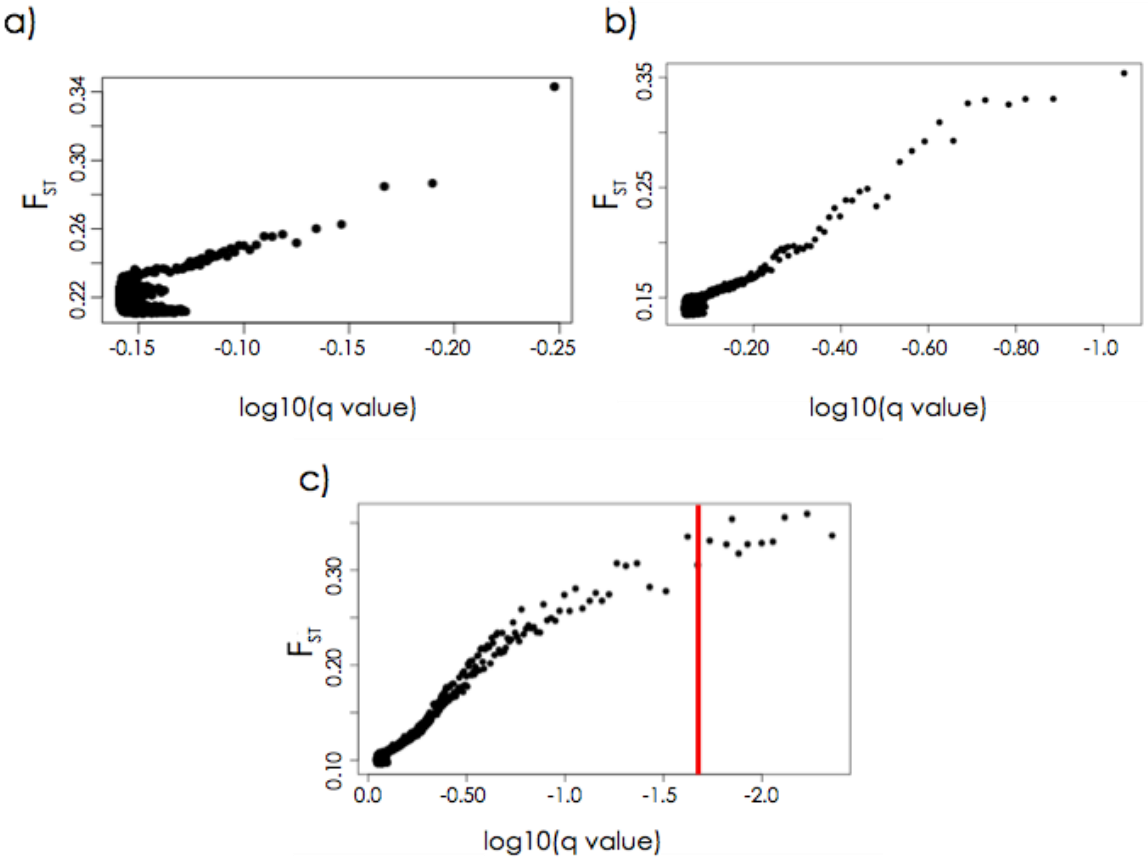

Figure 3.3 F_{ST} outlier results
Tests for F_{ST} outliers in data sets A (a), B (b), and C (c). A red line denotes the false discovery rate (q-value).

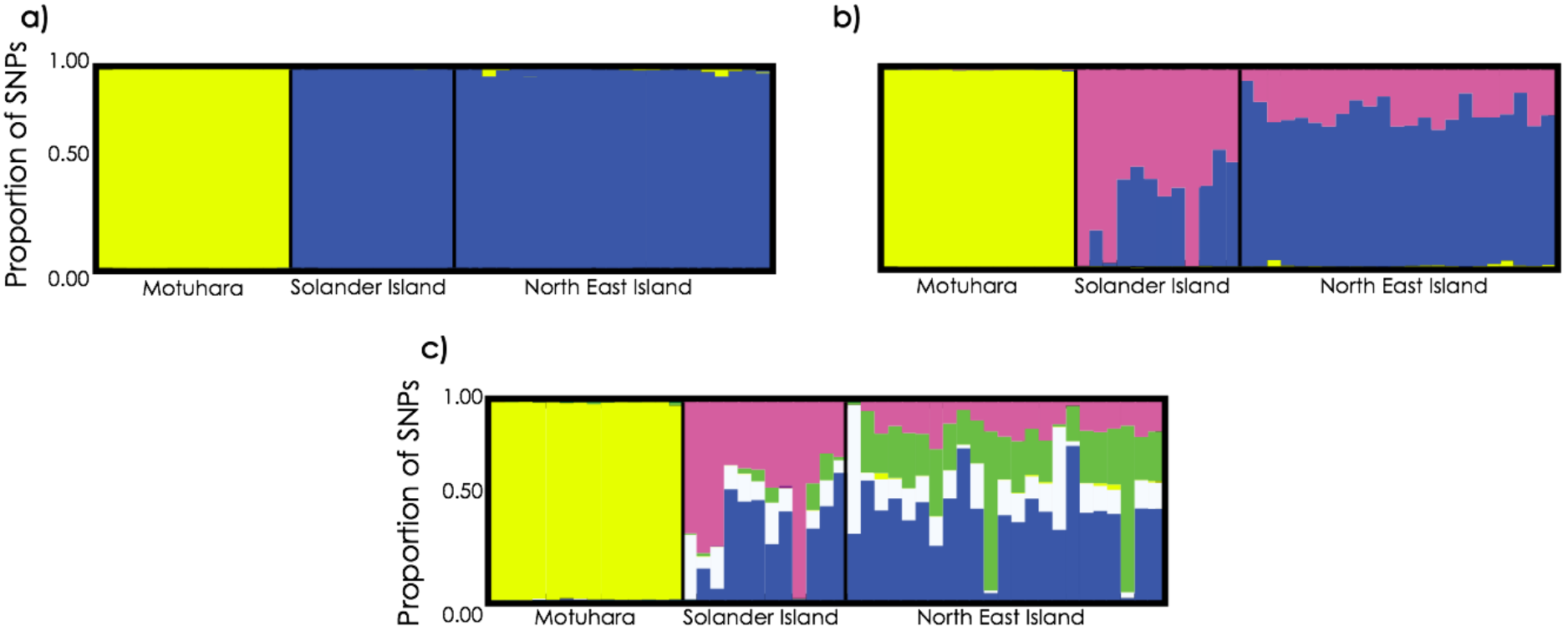

Figure 3.4 Structure plots for Buller's Albatross ssp.
STRUCTURE plots were constructed using GBS data set C (*see* **Table 3.2**). One Northern Buller's Albatross colony (*Thalassarche bulleri platei*; Motuhara) and two Southern Buller's Albatross colonies (*T. b. bulleri*; Solander Island, North East Island) are represented.

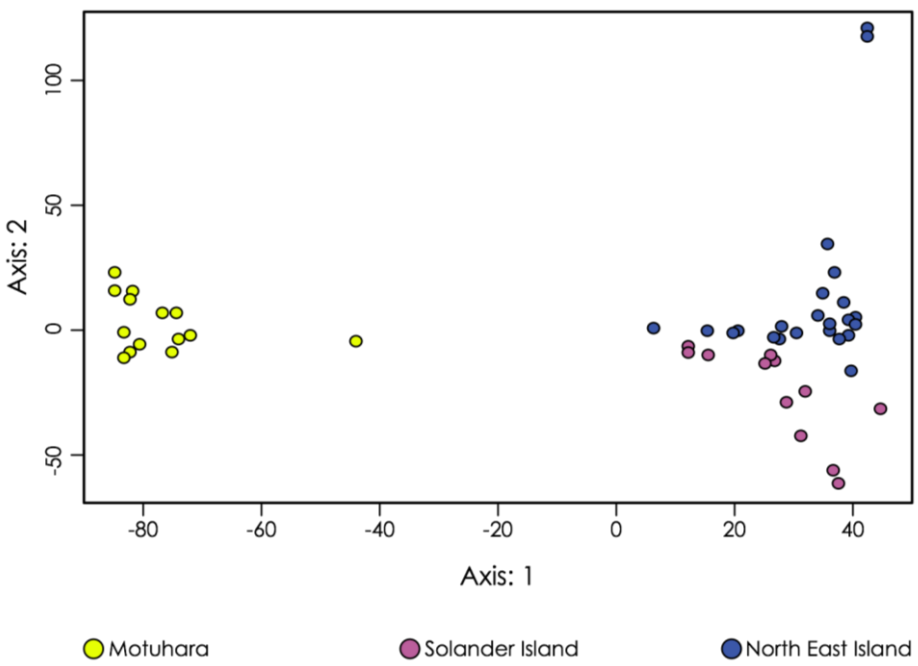

Figure 3.5 Principal Coordinate Analysis (PCA) of three Buller's Albatross ssp. breeding colonies
The genetic differentiation between one Northern Buller's Albatross colony, and two Southern Buller's Albatross colonies were visualised with a PCA. Where Northern Buller's Albatross = Motuhara, and Southern Buller's Albatross = Solander Island, North East Island.

Discussion

The results of this study showed that there does not appear to be a significant amount of gene flow between Northern and Southern Buller's albatross populations. While estimates of heterozygosity and π were consistent across data sets and taxon; pairwise F_{ST} values between taxa and each colony were significant (pairwise $F_{ST} \approx 0.018 - 0.122$). In addition, admixture analyses consistently showed two primary clusters that corresponded to taxon (Figure 3.4). The presence of F_{ST} outliers suggest that there were loci under directional selection, however these could not be mapped back to an annotated genome to identify their possible functions. Alternatively, the outlier loci could be caused by significant differentiation between the two groups as neutral and functional alleles differentiate over time with the evolutionary processes of mutation and genetic drift. Overall the analyses of the GBS data were consistent with the observation that Northern and Southern Buller's albatrosses are reproductively distinct groups (*see* Chapter 2).

GBS data output

The general lack of sample depth has led to a bias against heterozygous alleles within this data set (Figure 3.1). This bias could have resulted from a number of factors. Namely, sample quality, restriction enzyme choice, and the lack of a reference genome. Buller's Albatross ssp. breeding colonies occur on remote islands, some of which are notoriously difficult to access. The samples that were available for this project were collected in the early 90's and early 00's. Sample degradation (influenced by length of time in storage, the storage history, and the collection method) appeared to be significant contributing factor to the quality of DNA sequencing results.

Protocols designed to optimise marker recovery often consider genome size and complexity. Because the genome size for Buller's Albatross ssp. is unknown, restriction enzyme choice was determined with the high and low size estimates for the avian genomes (Gregory 2017, www.genomesize.com), and the gel electrophoresis analysis of the DNA samples after digested with a restriction enzyme (Appendix IV). The restriction enzyme *Pst*I was chosen because it produced the largest fragments. It was our hope that a conservative approach to digestion would increase sample depth. However, the genome composition of Buller's Albatross ssp. may have affected the distribution and consistency of cut sites. This potentially resulted in a concentration of reads in a particular location of the genome. In addition, many sequence reads were contaminated with the adapter sequence. This occurred when reads were less than the expected 101 bp and thus the adapter sequence was included into sample reads. The contamination of sample reads with adapter sequences may be due to the inappropriate annealing of adapters to restriction enzyme cut sites. While reads could be trimmed to remove the adaptor sequence and the alignment of reads, many reads were lost due to remaining adapter content. An avian specific GBS protocol would likely have increased the depth of sample reads. In aggregate, sample degradation and enzyme choice likely contributed to the overall low sample stack depth within this data set.

To investigate the effects of low sample stack depth on diversity estimates, the threshold for minimum per locus sample depth was varied from

–m = 4 to –m = 20 (Figure 3.1). Heterozygosity estimates significantly increased with minimum depth thresholds, yet plateaued around –m = 10. While the minimum sample depth used in genotyping was –m = 4, the mean sample depth of individuals retained for analysis was –m = 8. In short, there is a bias towards homozygous sites and as a result genetic diversity indices may be under estimated. While the number of homozygous sites likely reduced subsequent heterozygosity and π estimates, the mean sample depth met minimum thresholds for heterozygous genotypes.

Three different filtering protocols were assessed to investigate the effect of SNP filtering settings on diversity and differentiation estimates. The frequency of per locus π, and per locus F_{ST} values were estimated across data sets A, B & C. Distributions of per locus π were similar across data sets A, B & C (Appendix V), although the distributions of per site F_{ST} estimates varied (Figure 3.2). The removal of loci with excessively high heterozygosity and minor alleles reduced the number of spurious genotypes in the data set (Figure 3.2). The incorrect pairing of paralogs may have caused the very high levels of heterozygosity observed at some loci, whereas sequencing error might have been a contributing factor to the high number of minor alleles. The removal of loci with high H or minor alleles provided for a better comparison between the northern and southern taxa.

The low read depth that was used in the data set introduced a pipeline bias against heterozygous sites, which has implications for estimates of heterozygosity based diversity indices. For example, heterozygosity estimates all sites were similar across data sets A, B & C. However, when considering variant sites only, estimates increased as SNP filtering protocols became increasingly restrictive (Table 3.1). Overall F_{ST} values mirrored this trend, with values increasing from $F_{ST} = 0.018$ in data set A to $F_{ST} = 0.038$ in data set C. This is likely due to the removal of spurious alleles and loci as filtering protocols became increasingly restrictive. Nevertheless, heterozygosity estimates may be underestimated within this data set. For example, of the 17,236 putative loci in data set C, 5,739 were found to depart from HWE expectations.

Patterns of genetic diversity

Understanding patterns of genetic diversity within and among populations can provide insights into the overall evolutionary potential of populations (Hughes *et al.* 2008). Measures of genomic differentiation were consistently small across data sets; whereas pairwise F_{ST} calculations were significantly larger than overall F_{ST} estimates. This is because only the variant SNPs were considered in pairwise comparisons, and as a result the homogenising effects of invariant sites were removed. Pairwise F_{ST} values demonstrated moderate differentiation between taxa and small, but significant differentiation between colonies within a taxon (Table 3.3). A previous study into the population structure among colonies of Southern Buller's albatrosses found no evidence for population substructure (van Bekkum *et al.* 2006), but that finding was not consistent with the results of the GBS analysis.

The GBS sample of Northern Buller's albatrosses was only from individuals of a single colony; as such a test for connectivity among northern colonies could not be conducted and compared to the findings from Southern Buller's albatrosses. In addition, it is important to note that the SNP data sets were filtered to optimise taxa level comparisons. As such, some loci unique to each taxon were excluded from analyses. This may have decreased the statistical power for colony level comparisons. SNP filtering protocols specific to colony level comparisons would be need to properly assess the level for difference between those groups.

Without the benefit of a reference genome, we are unable to identify the genome location of the loci found to be F_{ST} outliers, or the genes that they are located near. Further, due to the significant differentiation between Northern and Southern Buller's albatrosses, this may be the result of divergence of neutral loci, rather than the result of differential selection. Admixture analyses and PCA visualization demonstrated a strong signal differentiating northern and southern taxa across three different models (Figure 3.4, Figure 3.5). The seemingly obvious difference in the size of PCA groups is likely reflective of sampling, Northern Buller's albatrosses are only represented by a single colony

while Southern Buller's albatrosses are represented by a larger sample size of two colonies.

Maintenance of genetic differentiation

A review conducted by Munro and Burg (2017) found that glacial refugia have shaped contemporary patterns of population structure in Southern Ocean seabirds. These patterns may have been maintained by behavioural and biological constraints. Determining whether philopatric behaviour influences the level of genetic differentiation can be difficult. Despite a number of taxa exhibiting high levels of natal philopatry, migrants have been observed moving between conspecific colonies and occasionally hybridisation occurs among closely related taxa such as Sooty and Laysan albatrosses (Fisher 1972; Rohwer *et al.* 2014).

Migrants and range expansions have been observed in a number of philopatric taxa (Harris 1973; Prince *et al.* 1994; Sagar *et al.* 1998; Weimerskirch *et al.* 1997). Natal philopatry is generally believed to be a primary driver in population differentiation for many seabirds. However, the relative significance of natal philopatry in maintaining genetic differentiation may be overstated (Coulson 2016). Variation in breeding phenology may be a primary mechanism maintaining species boundaries and differentiation among some populations. In cases where two taxa are experiencing secondary contact, the probably of reproductive hybridisation can be reduced by an asynchronous breeding seasons (Brown *et al.* 2015). In addition, there is some evidence suggesting that the breeding phenology of hybrid offspring may not align with either parent taxon. As a result, an asynchronous breeding season can result in reduced gene flow between taxa, and reduce the reproductive success of hybrids, which has been reported for Giant petrels and Antipodean albatrosses (Bost *et al.* 1992; Henry & Day 2005; Jouventin *et al.* 2006; Overeem *et al.* 2008). However, it is important to note that few closely related taxa have breeding seasons as distinct as Northern and Southern Buller's albatrosses. The egg lay times of the two Buller's Albatross ssp. are three months apart, which most likely isolates northern and southern populations. Due to the significant investment in care, and monogamous breeding system, it is unlikely

that Buller's Albatross ssp. would make two breeding attempts at different colonies.

The lack of an observed admixture between Buller's Albatross subspecies, and the high level of differentiation found using the mitochondrial control region (*see* Chapter 2), indicates that there is a lack of substantial historical and contemporary gene flow between the groups. Recent range expansions have been observed in a number of taxa (Harris 1973; Prince *et al.* 1994; Sagar *et al.* 1998; Weimerskirch *et al.* 1997), with some colonies experiencing multiple colonisation events by otherwise philopatric groups. Thus, it is unlikely that natal philopatry alone can be attributed to the maintenance of differentiation between northern and southern Buller's Albatross ssp. The relative significance of site fidelity and asynchronous breeding phenology in maintaining genetic isolation between Buller's Albatross ssp. cannot be explored with a genetic data set alone.

The degree of environmental influence vs. biological constraints in breeding phenology has not been explored to a great extent within albatrosses. Some evidence indicates that the at-sea distribution and subsequent exposure to ocean temperatures influences the breeding phenology of seabird populations (Overeem *et al.* 2008). In contrast, the maligned timing of some hybrids indicates that breeding phenology may be biologically regulated (Brown *et al.* 2015). If so, the maintenance of genetic differentiation between Northern and Southern Buller's albatrosses may be female mediated as the stages of egg development occur within a particular time frame. Further, Northern Buller's laying time is more tightly bounded than that observed in Southern Buller's (Appendix III). Tracking data for the Northern Buller's albatrosses may be able to elucidate this relationship by confirming the at-sea distribution of this taxon and may provide insights into the relative role of environmental factors and breeding phenology.

The result of this research identified genetic isolation between Northern and Southern Buller's albatrosses and indicates that there is some population substructure between the Southern Buller's Albatross colonies. Similar to other seabird populations, natal philopatry may be limiting dispersal between

colonies within the southern taxon. The rate and direction of gene flow between the two groups could be helpful for conservation management, because it could be used to identify whether a particular population is a higher source of migrants and enable it to have a higher conservation priority. Alternatively, if the rate of exchange is similar between two populations, then they could be managed as a single group. Finally, if no gene flow is detected between the two, they could be considered two evolutionarily significant units with potential source and sink pops within each (Moritz 1994b; Pulliam 1988).

This SNP data could be examined in more detail to investigate the influence of low sample depth on differentiation and diversity estimates. Although a reference genome for Procellariiformes is currently unavailable, mapping back to alternative high coverage avian genomes may help resolve some of the ambiguities of gametic linkage. Future work with this data set should include mapping loci back to reference genomes *as per* Dierickx *et al.* (2015) to increase confidence in genotyping. This may be helpful for determining if outlier loci correspond with functional genes.

CHAPTER 4 GENERAL DISCUSSION

In recognition of the threatened status of most Procellariiformes species, many countries have committed to conservation initiatives, which include the Agreement on the Conservation of Albatrosses and Petrels (ACAP) and the International Union for Conservation of Nature (IUCN). How these international reference groups characterize and distinguish species will, in turn, determine how conservation agencies organise and prioritise their efforts (Croxall et al. 2012). Four Procellariiformes genera are universally recognised, Diomedea, Phoebastria, Phoebetria and Thalassarche (Brooke 2002), but ranks of various species and groups within these genera have not been fully resolved (see Burg & Croxall 2004). The taxonomy within albatrosses is contentious, which can make the estimation of the size and number of breeding groups difficult to determine (*see* Abbott & Double 2003a; Alderman et al. 2005).

For some albatross species fishing related mortality is a primary source of population disturbance (Lewison & Crowder 2003; Tuck *et al*. 2001; Zador *et al*. 2008). However, it is difficult to estimate the effect of mitigation measures on seabird bycatch, as monitoring efforts have historically been poor (Bull 2009). The offshore islands surrounding New Zealand are a biodiversity hotspot for seabird taxa (*see* Croxall *et al.* 2012), many of which do not breed elsewhere in the world. In recognition of New Zealand's unique status in seabird biodiversity, a National Plan of Action to Reduce the Incidental Catch of Seabirds in New Zealand Fisheries (NZPA) was drafted in 2003 and ratified in 2004. The NZPA has the specific aims of ensuring that the long-term viability of protected seabird species is not hindered by incidental interactions in NZ fisheries or by NZ flagged vessels operating in foreign waters; and to consider the financial implications as well as advances in technology and knowledge to further reduce incidental catch of protected seabird species. Specific fisheries recognised for high seabird mortality within NZ commercial fishing operations include longline and trawling (Waugh *et al.* 2008). This prompted the implementation of on-board observer programs to more

accurately assess wildlife interactions in commercial fisheries and enforcement of industry best practices as per the NZPA. However, the inconsistent use and enforcement of mitigation measures in international waters may still result in avoidable seabird mortality. This gap in mitigation occurs because vessels in international jurisdictions are not required to follow the agreed protocols, or the absence of explicit performance standards (Gilman *et al.* 2013).

Many Southern Ocean seabirds have trans-oceanic or circumpolar distributions (*see* del Hoyo & Collar 2014). As a result, they are exposed to a wide range and number of fisheries management areas (Croxall *et al.* 2012). The presence of independent observers appears to increase the likelihood of enforcement and the accurate documentation of seabird interaction. However, on-board observer programs and onshore necropsy of seabird bycatch continues to be limited by the ability to precisely identify the species and population-of-origin for individual birds. This is important because reproductively isolated seabird populations tend to share feeding areas but the proportions of species and populations will often vary a lot in time and space (*see* Burg & Croxall 2001; Friesen 2015; Friesen *et al*. 2007). As such, some populations may suffer more harm at different places and at different times of the year.

The relative portions of populations found in the bycatch may have significant implications for the long-term persistence of some populations and species (Hughes *et al.* 2008). Unless there is certainty about the numbers, it is difficult determine the risk levels of each species and population. Buller's Albatross (*Thalassarche bulleri* ssp.) are currently recorded in the top five fisheries interactions within the New Zealand EEZ (Clemens-Seely *et al.* 2014a, b; Clemens-Seely & Osk Hjorvarsdottir 2016). However, the relative impact on two subspecies the Northern Buller's Albatross (*T. b. platei*) and the Southern Buller's Albatross (*T. b. bulleri*) remained unresolved even with an on-board observer and a necropsy program.

Genetics and seabird bycatch

Determining the relative impact of fisheries-related mortality on a particular species or subspecies of albatross with on-board observers alone can

be challenging due to the limited taxonomic skills of many observers. Further, morphology-based identification by experienced observers would not provide population level resolution in some seabirds (*see* Dierickx *et al.* 2015 *for partial example*). A DNA-based approach has demonstrated its usefulness as a tool for identifying illegally trafficked animal products (*see* Dawnay *et al.* 2007), and to assign provenance to species from highly structured populations (Abbott et al. 2006; Burg 2007; Moritz 1994). Further, by characterising the relative diversity within and between populations, the prioritisation of conservation may be focused on the relative diversity lost to bycatch, and whether populations are disproportionately represented within particular fisheries.

The application of genetic data to estimate the relative proportion of Procellariiformes represented in bycatch has only been applied to a few taxa (Burg 2007; Edwards *et al.* 2001; Inoue *et al.* 2015). However, the relative impacts of fisheries interactions on a particular population or group of breeding colonies remains largely unknown (*see* Abbott *et al.* 2006; Baker *et al.* 2007; Gomez-Diaz & Gonzales-Soli 2007; Walsh & Edwards 2005 *for partial examples*). When choosing genetic markers for a diagnostic tool, it is important to note the breadth and scope of the proposed research. Mitochondrial and nuclear DNA markers can provide varying degrees of resolution and address historical processes, or contemporary patterns of gene flow (Avise 2000). The mitochondrial DNA gene cytochrome *b* has been utilised in a number of phylogenetic studies aiming to resolve the taxonomy of albatrosses (Chambers et al. 2009; Nunn et al. 1996; Penhallurick & Wink 2004). This particular gene was found to be relatively conserved as pairwise differences between species were somewhat small (Chambers et al. 2009). A pilot study for this thesis research found that the mitochondrial gene cytochrome oxidase subunit I (COI) is likely to be similarly conserved within the family (*data not presented*). However, analysis of the hypervariable control region, Domain II (CRII), captured the population level differences for Buller's Albatross ssp. (*see* Chapter 2). This is consistent with a number of studies have had success with using CRII in distinguishing population level differences within albatrosses (Abbott et al. 2006; Burg 2007; Walsh & Edwards 2005). The results of this

study showed that CRII could be used as an effective genetic marker to determine the species and sub-species of a bycaught sample.

Mitochondrial markers alone may provide insights into the genetic connectivity between Buller's Albatross ssp. Yet due to the maternal inheritance of mtDNA, this marker alone would not detect the degree of hybridisation and potential introgression between two species (Avise 2000). The bi-parental inheritance of nuclear markers enables the detection of contemporary gene flow patterns and the ability to infer the connectivity among populations (Avise 2000). Microsatellite markers are a common choice in population genetic studies (*see* Edwards *et al.* 2001). However, the application of microsatellite markers to multiple species may not be possible. This was the case when microsatellite DNA markers that were originally designed for Wandering (*Diomedea exulans*) and Shy albatrosses (*Thalassarche cauta*) were trialled on Buller's Albatross ssp. Of the 14 markers assessed, 8 successfully amplified, of which, only 3 provided clear alleles for scoring and downstream analyses. A better sample of variable loci would be needed to provide the appropriate level of statistical power to estimate the genetic connectivity between Buller's Albatross ssp. (Barraclough & Nee 2001; Takezaki & Nei 1996).

As an alternative to microsatellite markers, next-generation sequencing (NGS) methodologies can generate multiple marker types while simultaneously sampling 1,000's of loci throughout the genome (Baird *et al.* 2008; Elshire *et al.* 2011). One method is reduced representation genome sequencing approach, which encompasses Restriction Associated DNA sequencing (RAD-seq; Baird *et al.* 2008) and more generally the Genotyping by Sequencing (GBS; Eshire *et al.* 2011) methods. These methods are useful in species where prior knowledge of the genome is not readily available (Baird et al. 2008; Elshire et al. 2011), and can simultaneously identify markers and genotype individuals (Elshire et al. 2011).

To investigate the degree of admixture among Buller's Albatross ssp. a SNP data set was generated using GBS. This data set improved upon a previous microsatellite study assessing the degree of connectivity among

Southern Buller's Albatross colonies (van Bekkum *et al.* 2006). The increased resolution obtained with GBS showed that the two Southern Buller's Albatross colonies included within this research were significantly differentiated from one another (*see* Chapter 3). Yet, colonies within a taxon were still experiencing some level of admixture (*see* Appendix VI). In contrast, there was significant evidence to support the genetic isolation between Buller's Albatross ssp. Genome wide datasets are a powerful tool to investigate genetic connectivity between populations (Davey et al. 2011). The marker types discussed here are not an exhaustive list of available markers for population genetics. Due to the high statistical power and decreasing cost of NGS technologies, it is likely that its use will be increased in conservation and population genetic research.

Population structure

Past glaciations are believed to have largely influenced the contemporary population structure in Southern Ocean seabirds. As a result, speciation through range expansion is believed to have given rise to many of the taxa we see today (*see* Munro & Burg 2017 *for review*). There are a number of examples of range expansions within Procellariiformes, where natal philopatry is prevalent among taxa (Coulson 2002; Friesen *et al.* 2007). While there is little doubt that natal philopatry has some role in the differentiation among populations, it's effectiveness in maintaining genetic differentiation may be overstated (Coulson 2016).

Population connectivity and gene flow are indirectly inferred from population structure, of which there are multiple metrics. These include morphological (Smith & Friesen 2007), behavioural (at-sea distribution, Burg & Croxall 2001; variation in mate calls, Bretagnolle 1989) and biological (breeding phenology, Henry & Day 2005). Currently, there is an absence of published primary literature investigating diagnostic morphological traits for distinguishing between Buller's Albatross ssp. Behavioural variation between Buller's Albatross ssp. is similarly under studied. In addition, while the at-sea distribution of the southern taxon is well documented (BirdLife International 2004), it remains unknown for the northern taxon. The genetic assignment of

bycatch may indicate some temporal partitioning at-sea during the breeding seasons (Robertson *in prep*; Appendices VII & VIII). Continued assignment of bycatch within the New Zealand EEZ, and abroad, and tracking data for Northern Buller's albatrosses would resolve the at-sea distribution of Northern Buller's Albatross. It is possible that the two taxa do not vary geographically, but vary temporally.

The specific factors influencing the breeding phenology of seabirds remain unresolved due to contradictory evidence. There is some indication that breeding phenology may be influenced by environmental factors such as ocean temperatures and prey distribution (Overeem et al. 2008). However, this is contrasted by evidence that suggests breeding phenology is biologically constrained. Northern Giant Petrels (*Macronectes halli*) and Southern Giant Petrels (*M. giganteus*) are a good example of this. The breeding phenology of Giant Petrels spp. is asynchronous despite overlap in the foraging and breeding distributions of both taxa (Bourne & Warham 1966). Giant Petrel spp. breeding on Bird Island, South Georgia have an asynchronous breeding season with Northern Giant Petrel lay dates preceding that of Southern Giant Petrels (Brown et al. 2015). Hybridisation between Giant Petrel sp. was found to bias towards male Southern Giant Petrels pairing with the earlier breeding female Northern Giant Petrels (Brown et al. 2015).

If hybridisation were to occur between Buller's Albatross ssp., pairs would likely consist of female Northern Buller's albatrosses and male Sothern Buller's albatrosses, it is unlikely female albatrosses would shift lay dates by three months. As a result, the significant differentiation and maintenance of genetic isolation between Buller's Albatross ssp. may largely the result of asynchrony in breeding phenology. Further, the correlation of maternally inherited reproductive timing in Giant Petrels indicates that breeding phenology may be biologically constrained. First generation hybrids were recruited into their maternal breeding population, though attempts were generally later than that of the general Northern Giant Petrel population (Brown *et al.* 2015). The opportunity for reproductive success between Procellariiformes may be influenced by the degree of differentiation in breeding

seasons. Given significant differentiation in lay dates, the rate of hybridisation may not be equal among the two taxa, as females from the earlier breeding females pair with males who are more flexible in reproductive timing.

Many seabirds live highly specialised life histories as reproductive success is predicated upon males and females to returning to breeding colonies in a synchronised manner. No other closely related albatross taxa have breeding seasons as differentiated as Northern and Southern Buller's albatrosses. This differentiation in breeding seasons, coupled with the significant differentiation at CRII, and the lack of contemporary admixture between the two taxa suggest that Northern and Southern Buller's albatrosses are experiencing isolation by time (Hendry & Day 2005).

Implications for taxonomy

While this study did not intend to review the taxonomic status of Northern and Southern Buller's Albatross, it is hoped that these data will be used to inform any future taxonomic revisions. The two Southern Buller's Albatross colonies assessed in this study did not show significant historical isolation (*see* Chapter 2), and likely experience contemporary admixture (*see* Chapter 3). In contrast, differentiation between Northern and Southern Buller's albatrosses consistently demonstrated significant genetic isolation, this may be attributed to the highly differentiated breeding phenology of the two taxa, and as a result the two may be experiencing isolation-by-time (IBT). If isolation-by-time is taking place between Northern and Southern Buller's Albatross, it may have implications for the taxonomic status of the two taxa.

At times, morphological variation may not correspond with population differentiation or speciation (*see* Bickford *et al.* 2007), resulting in distinct species that appear the same based on external features. The advance of genetic methods has allowed for the identification and description of many cryptic species (Bickford *et al.* 2007), although this process still remains challenging. For example, when examining genetic differentiation between two allopatric populations, it can be difficult to determine if they are distinct species or two isolated populations (Bickford *et al.* 2007). Cryptic species are

more easily identified if samples are drawn from sympatric populations comprised of two reproductively isolated species as in Buller's Albatross ssp. The identification of cryptic species in sympatric populations is simplified by the fact that they have the opportunity to intermix, but do not. Cryptic speciation is a difficult pattern to discern within seabirds because there are no clear geographic barriers to gene flow among populations of Southern Ocean Procellariiformes (Munro & Burg 2017). Yet seabirds reproduce within the confines of discrete colonies. This can potentially lead to the incorrect assumption of allopatric speciation.

A holistic taxonomic approach has been advocated for Albatrosses recognised as distinct species (Chambers *et al.* 2009). As a result, a number of species are recognised despite the ability and the frequency in which hybridisation occurs after secondary contact (Brown *et al.* 2015; del Hoyo & Collar 2014; Moore *et al.* 2001). In cases where prolonged and consistent reproductive isolation is detected, and may be perpetuated between populations, a taxonomic review may be warranted. Further, although the sample size is small, the assignment of bycatch within this study indicates that Buller's Albatross ssp. vary temporally in their distribution in the New Zealand EEZ (Robertson *in prep*).

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APPENDIX I

Appendix Table 1 Information for samples caught as bycatch within the New Zealand EEZ

Necropsy No.	Date (dd/mm/yy)	Coordinates when captured	Sex	Genetic Assignment
000602	04/06/00	43°43'S, 177°18'E	Male	*Thalassarche bulleri platei*
000603	04/06/00	43°43'S, 177°18'E	Female	*Thalassarche bulleri platei*
000609	06/06/00	43°54'S, 174°49'E	Male	*Thalassarche bulleri bulleri*
000723	15/06/00	37°10'S, 179°13'E	Male	*Thalassarche bulleri platei*
000731	11/06/00	43°12'S, 175°55'E	Female	*Thalassarche bulleri bulleri*
000732	11/06/00	43°12'S, 175°55'E	Female	*Thalassarche bulleri bulleri*
000733	16/06/00	43°26'S, 174°12'E	Female	*Thalassarche bulleri bulleri*
000734	11/06/00	43°12'S, 175°55'E	Female	*Thalassarche bulleri bulleri*
000735	08/06/00	43°01'S, 174°58'E	Female	*Thalassarche bulleri bulleri*
011267	29/01/01	48°48'S, 166°42'E	Male	*Thalassarche bulleri bulleri*
011463	05/04/01	42°49'S, 177°16'E	Male	*Thalassarche bulleri platei*
011464	11/04/01	43°50'S, 176°08'E	Female	*Thalassarche bulleri bulleri*
011516	20/04/01	48°39'S, 167°20'E	Female	*Thalassarche bulleri bulleri*
011530	19/03/01	48°44'S, 167°31'E	Male	*Thalassarche bulleri bulleri*
011557	23/04/01	48°34'S, 167°33'E	Male	*Thalassarche bulleri bulleri*
011903	01/06/01	44°41'S, 166°15'E	Female	*Thalassarche bulleri bulleri*
011912	05/05/01	45°36'S, 165°46'E	Female	*Thalassarche bulleri bulleri*
011935	04/06/01	44°19'S, 166°52'E	Female	*Thalassarche bulleri bulleri*
011975	09/09/01	46°37'S, 166°10'E	Male	*Thalassarche bulleri bulleri*
012012	04/05/01	46°13'S, 165°41'E	Female	*Thalassarche bulleri bulleri*
012013	06/06/01	44°25'S, 166°45'E	Male	*Thalassarche bulleri bulleri*
012014	05/05/01	46°11'S, 165°55'E	Female	*Thalassarche bulleri bulleri*
022463	26/02/02	40°18'S, 177°51'E	Female	*Thalassarche bulleri platei*
022849	12/08/02	42°31'S, 170°26'E	Female	*Thalassarche bulleri bulleri*
033315	11/06/03	41°10'S, 174°50'E	Male	*Thalassarche bulleri bulleri*
033393	12/06/03	43°17'S, 166°15'E	Female	*Thalassarche bulleri bulleri*
043937	01/06/04	41°58'S, 169°43'E	Male	*Thalassarche bulleri bulleri*
054245	15/12/04	43°27'S, 176°04'W	Male	*Thalassarche bulleri platei*
100147	21/05/11	39°13'S, 178°41'E	Male	*Thalassarche bulleri bulleri*
100154	23/05/11	44°55'S, 165°01'E	Male	*Thalassarche bulleri bulleri*
100173	18/05/11	44°52'S, 165°04'E	Male	*Thalassarche bulleri bulleri*
100174	28/05/11	46°46'S, 170°02'E	Female	*Thalassarche bulleri bulleri*
100595	26/03/12	46°28'S, 170°00'E	Male	*Thalassarche bulleri bulleri*
100599	20/03/12	43°16'S, 176°31'W	Male	*Thalassarche bulleri platei*
100692	07/05/12	46°07'S, 164°55'E	Female	*Thalassarche bulleri bulleri*
100693	27/04/12	50°45'S, 166°56'E	Male	*Thalassarche bulleri bulleri*
100698	06/05/12	46°04'S, 165°03'E	Female	*Thalassarche bulleri bulleri*
100703	28/05/12	50°28'S, 167°34'E	Male	*Thalassarche bulleri bulleri*
100708	26/05/12	42°37'S, 170°37'E	Female	*Thalassarche bulleri bulleri*
100732	01/07/12	43°37'S, 174°04'E	Male	*Thalassarche bulleri bulleri*
100748	27/07/12	42°34'S, 170°19'E	Female	*Thalassarche bulleri bulleri*

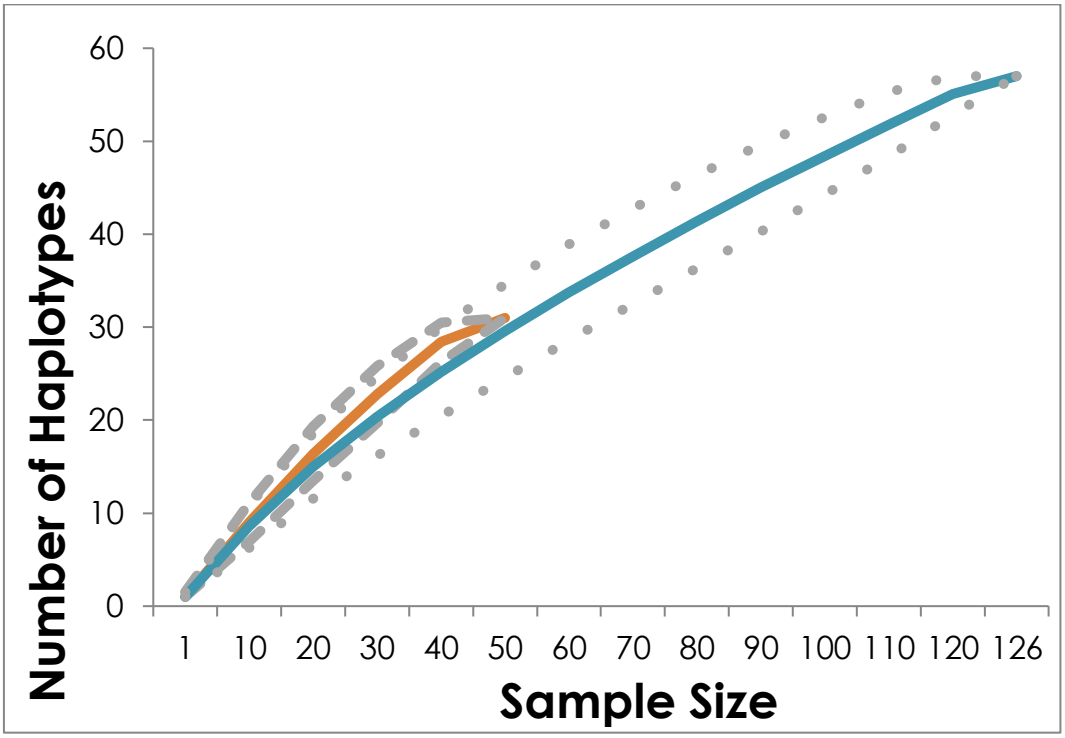
Necropsy No.	Date (dd/mm/yy)	Coordinates when captured	Sex	Genetic Assignment
100781	20/09/12	49°04'S, 166°34'E	Male	*Thalassarche bulleri bulleri*
101181	08/04/13	48°52'S, 166°41'E	Male	*Thalassarche bulleri bulleri*
101186	17/03/13	50°17'S, 166°40'E	Male	*Thalassarche bulleri bulleri*
101313	11/06/13	50°55'S, 167°14'E	Male	*Thalassarche bulleri bulleri*
101314	18/04/13	48°48'S, 166°42'E	Female	*Thalassarche bulleri bulleri*
101315	16/05/13	43°51'S, 174°47'E	Female	*Thalassarche bulleri bulleri*
101323	18/04/13	48°46'S, 166°35'E	Female	*Thalassarche bulleri bulleri*
101324	02/06/13	46°08'S, 170°48'E	Male	*Thalassarche bulleri bulleri*
101325	19/05/13	48°51'S, 167°02'E	Male	*Thalassarche bulleri bulleri*
101331	05/06/13	41°45'S, 170°25'E	Female	*Thalassarche bulleri bulleri*
101334	24/06/13	43°55'S, 168°03'E	Female	*Thalassarche bulleri bulleri*
101337	04/05/13	49°21'S, 168°34'E	Female	*Thalassarche bulleri bulleri*
101339	23/06/13	42°35'S, 170°19'E	Male	*Thalassarche bulleri bulleri*
101345	30/05/13	33°42'S, 174°16'E	Male	*Thalassarche bulleri platei*
101346	27/05/13	33°43'S, 174°16'E	Female	*Thalassarche bulleri bulleri*
101348	25/04/13	48°38'S, 166°21'E	Female	*Thalassarche bulleri bulleri*
101351	01/06/13	43°18'S, 174°16'E	Male	*Thalassarche bulleri bulleri*
101392	17/08/13	42°33'S, 170°24'E	Male	*Thalassarche bulleri bulleri*
101393	17/08/13	42°33'S, 170°24'E	Male	*Thalassarche bulleri bulleri*
101395	18/07/13	42°35'S, 170°18'E	Male	*Thalassarche bulleri bulleri*
101397	09/07/13	42°07'S, 170°28'E	Female	*Thalassarche bulleri bulleri*
101695	25/09/13	49°00'S, 166°32'E	Male	*Thalassarche bulleri bulleri*
101728	22/11/13	33°03'S, 173°48'E	Male	*Thalassarche bulleri platei*
101749	30/12/13	44°27'S, 178°40'E	Male	*Thalassarche bulleri platei*
101753	31/12/13	44°09'S, 176°58'E	Male	*Thalassarche bulleri platei*
101862	16/04/14	42°57'S, 179°14'E	Male	*Thalassarche bulleri platei*
101872	09/05/14	45°16'S, 165°14'E	Male	*Thalassarche bulleri bulleri*
101926	23/05/14	44°01'S, 174°40'E	Female	*Thalassarche bulleri bulleri*
102040	27/05/14	48°47'S, 168°58'E	Female	*Thalassarche bulleri bulleri*
102043	13/06/14	42°32'S, 170°25'E	Male	*Thalassarche bulleri bulleri*
102047	01/06/14	42°42'S, 169°27'E	Male	*Thalassarche bulleri bulleri*
102049	16/05/14	44°40'S, 165°45'E	Female	*Thalassarche bulleri bulleri*
102053	03/06/14	42°29'S, 170°17'E	Male	*Thalassarche bulleri bulleri*
102056	18/05/14	47°49'S, 169°02'E	Male	*Thalassarche bulleri bulleri*
102058	13/06/14	43°01'S, 166°42'E	Female	*Thalassarche bulleri bulleri*
102061	11/06/14	43°08'S, 166°13'E	Female	*Thalassarche bulleri bulleri*
102070	26/07/14	42°14'S, 170°34'E	Female	*Thalassarche bulleri bulleri*
102397	13/08/14	41°12'S, 170°48'E	Female	*Thalassarche bulleri bulleri*
102611	05/02/15	43°20'S, 176°12'W	Male	*Thalassarche bulleri platei*
102617	27/01/15	43°31'S 177°20'W	Male	*Thalassarche bulleri platei*
103083	27/05/15	43°33'S, 173°56'E	Male	*Thalassarche bulleri bulleri*
103084	08/06/15	43°45'S, 175°47'W	Male	*Thalassarche bulleri platei*
103086	06/06/15	43°44'S, 175°48'W	Male	*Thalassarche bulleri platei*
103092	15/05/15	49°21'S, 168°28'E	Male	*Thalassarche bulleri bulleri*
103095	09/06/15	43°45'S, 175°47'W	Male	*Thalassarche bulleri platei*
103110	21/09/15	46°25'S, 166°19'E	Male	*Thalassarche bulleri bulleri*
103112	11/07/15	36°55'S, 178°40'E	Male	*Thalassarche bulleri bulleri*

Necropsy No.	Date (dd/mm/yy)	Coordinates when captured	Sex	Genetic Assignment
103116	31/07/15	41°56'S, 170°35'E	Male	*Thalassarche bulleri bulleri*
103454	29/03/16	50°25'S, 167°25'E	Male	*Thalassarche bulleri bulleri*
103458	24/02/16	48°39'S, 167°41'E	Male	*Thalassarche bulleri bulleri*
103466	22/03/16	50°06'S, 166°13'E	Male	*Thalassarche bulleri bulleri*
103544	26/05/16	43°56'S, 174°21'E	Female	*Thalassarche bulleri bulleri*
103550	14/06/16	39°01'S, 178°30'E	Female	*Thalassarche bulleri platei*
103555	17/06/16	46°44'S, 176°00'E	Male	*Thalassarche bulleri bulleri*
103563	10/06/16	43°29'S, 174°30'E	Male	*Thalassarche bulleri platei*
990500	28/07/99	36°30'S, 178°51'E	Female	*Thalassarche bulleri bulleri*

APPENDIX II

Appendix Table 2 Hierarchical analysis of molecular variance (AMOVA) calculated with haplotype frequency

Source of Variation	D.F.	Sum of Squares	Variance Components	Percentage of variance	*F*-statistics	*p*-value
Among Regions	1	0.952	0.01506	3.02	*F*CT = 0.030	0.334
Among Populations within Regions	2	0.916	-0.00184	-0.37	*F*SC = -0.004	0.581
Within Populations	69	33.503	0.48555	97.35	*F*ST = 0.027	0.014
Total	72	35.37				



Appendix Figure 1 Rarefaction analysis

An analysis of rarefaction conducted with pooled samples of known and assigned bycatch. Where the orange line represents Northern Buller's Albatross sampled from Motuhara, Rangitatahi and assigned bycatch and the blue line represents Southern Buller's Albatross sampled from North East Island, Solander Island and assigned bycatch.

Appendix Table 3 Preliminary comparison of Buller's Albatross ssp. breeding attributes

A preliminary comparison of Northern Buller's albatrosses breeding at Motuhara and Rangitatahi islands in the Chatham Islands group, and Southern Buller's albatrosses breeding at The Snares and Solander Islands.

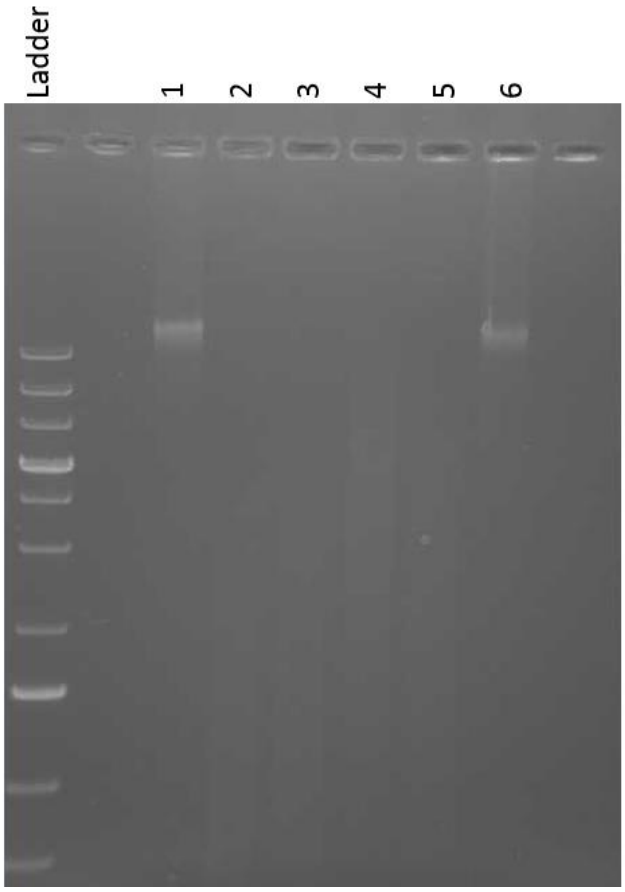
C. J. R. Robertson – Compiled Appendix for Jana Wold MSc thesis, March 2017

Activity	Northern Buller's' [1]	Southern Buller's' [2]
First recorded return to breeding colony	6-Sep	6-Dec
Range of egg laying	27 October - 23 November	26 December - 29 February
Peak laying period	10 - 11 November	21 - 26 January
Length of egg laying period (days)	28 days (80% of laying = 21 days)	43-- 62 days (80% of laying = 26 days)
Size of single egg	100.01 x 64.6 mm (n = 162)	103.5 x 64.9 mm (n = 509)
Mean length of incubation	68.8 days (66 - 70 days)	69.1 days (67 - 75 days)
Mean incubation shift after 1st shift	3.5 days (range 1 -11 days)	10.8 days (3 - 22 days)
Mean brooding shift	1.6 days (range 1 - 10 days)	2.1 to 2.7 days (range 1 - 12 days)
Brooding / guard stage length	23 - 28 days	23 - 25 days
Fledging period	Estimated 120 - 160 days	147 - 189 days, mean 167
Fledging dates	Late May - early July	22 August - 23 October

[1] CJR Roberston unpublished field studies data 1973 - 76, 1994 - 97

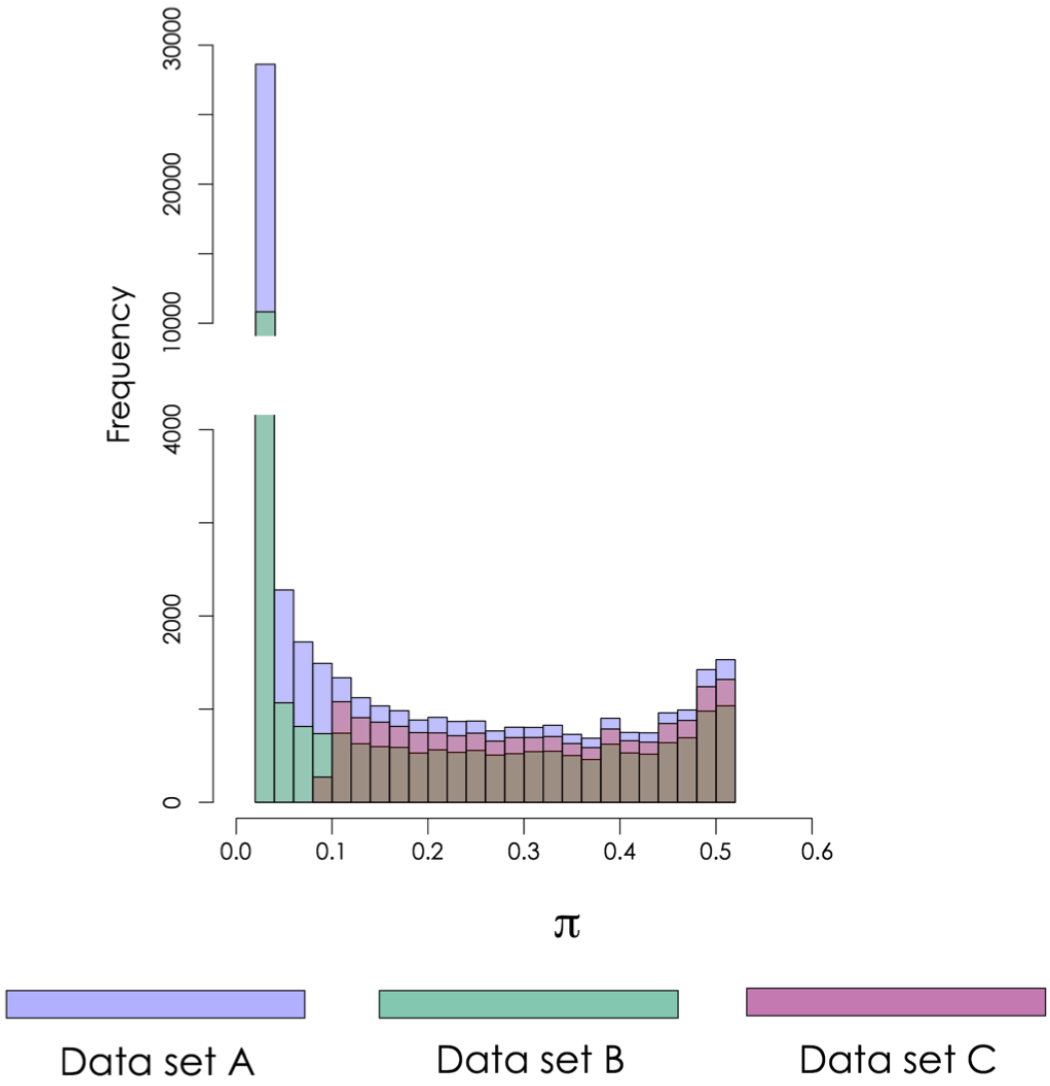
[2] Sagar, P. M. & Warham, J. 1998 Albatross Biology & Conservation pg 92 - 98

APPENDIX IV



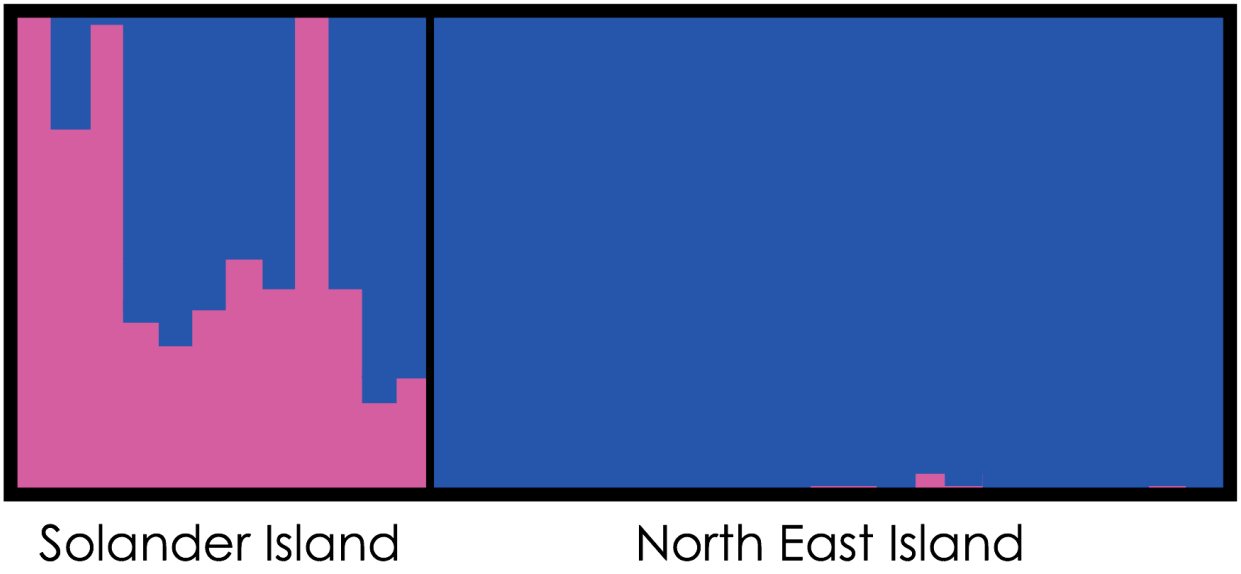
Appendix Figure 2 Size estimates of restriction enzyme products
Gel of restriction enzyme digestions. Fragment size and concentration were estimated against a GeneRuler 1 kb DNA ladder. Where lane 1 = Negative control (no restriction enzyme added to reaction); lane 2 = ApeKl; lane 3 = ApeKl/Mspl; Lane 4 = Pstl; lane 5 = Pstl/Mspl; lane 6 = undigested DNA. Image courtesy of AgResearch.

APPENDIX V



Appendix Figure 3 Comparisons of π between data sets A – C
Data sets A – C are inclusive of both Northern and Southern Buller's albatrosses

APPENDIX VI



Appendix Figure 4 STRUCTURE plot of two Southern Buller's colonies
Admixture analysis of the two Southern Buller's Albatross colonies examined in this research. The presence of 2 distinct clusters within Southern Buller's Albatross colonies was favored by K = 2. Philopatric behavior is likely leading to population structure between colonies on Solander Island and North East Island.

Appendix Table 4 Monthly distribution of Buller's Albatross ssp. bycatch from 1996 - 2016

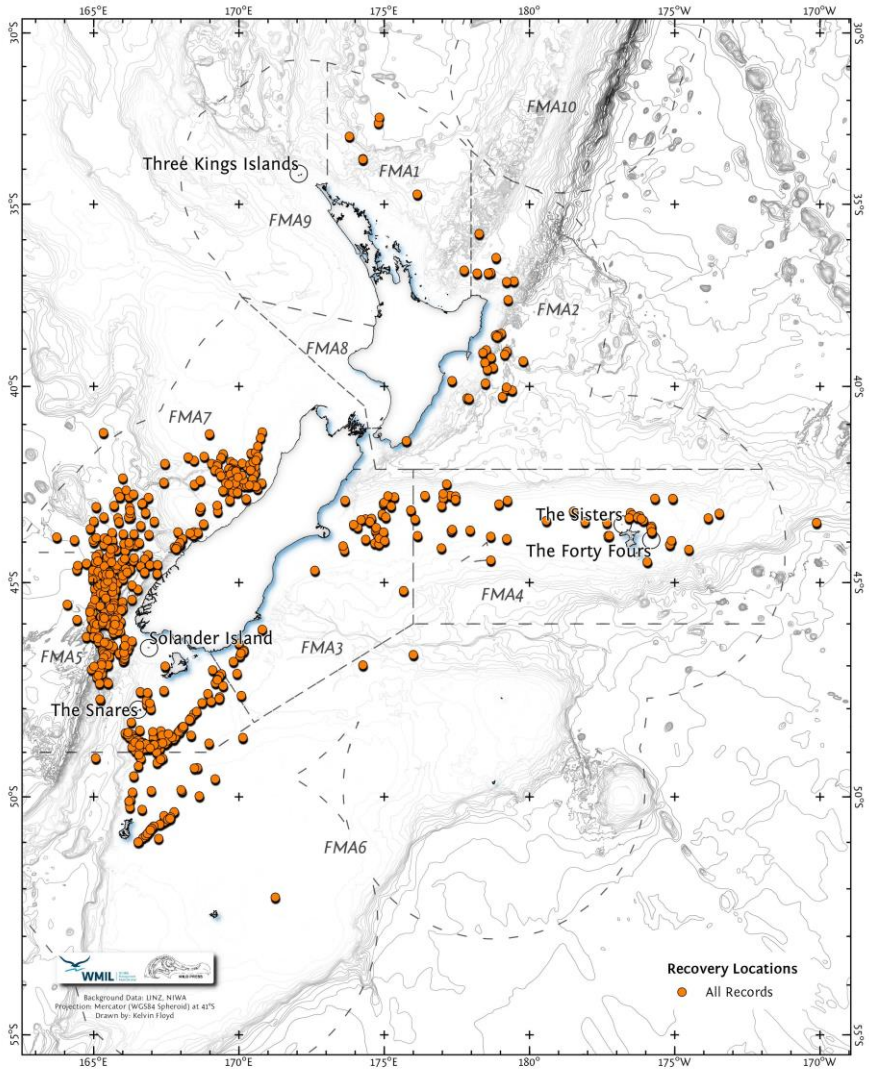
Table courtesy of Chris J. R. Robertson

Groupings of samples	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Totals
1996 - 2005 ALL CAUGHT	5	7	8	44	52	54	8	5	2	1	1	3	190
2005 - 2010 ALL CAUGHT	1	6	4	51	60	27	11	4					164
2010 - 2016 ALL CAUGHT	2	6	12	32	83	65	23	4	3	2	2	2	236
TOTAL													**590**
'NORTHERN' DNA	1	2	1	2	1	8					1	3	19
'SOUTHERN' DNA		1	5	8	24	24	8	4	4				78
TOTAL DNA													97

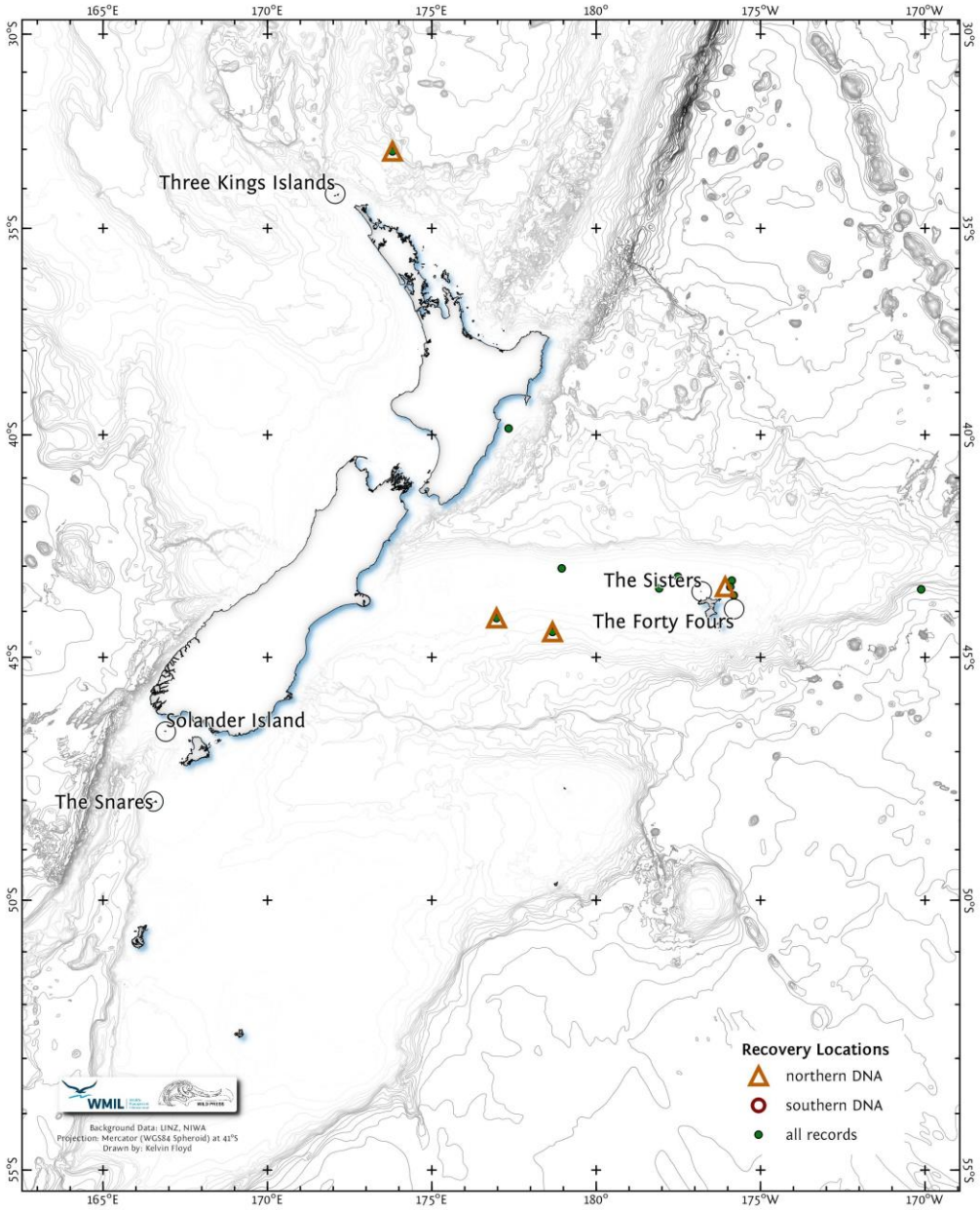
Monthly distribution of 590 Buller's albatross ssp., obtained from observed fisheries bycatch in New Zealand waters from 1996-2016.
The 97 analysed DNA samples came only from a total of 426 birds obtained in the periods 1996-2005 and 2010-2016.
The 1996-2005 period yielded 6 'northern' and 22 'southern DNA results.
The 2010-2016 period yielded 13 'northern' and 56 'southern' DNA results.
(C.J.R.Robertson analysis. pers. comm. 2/4/2017)

APPENDIX VIII

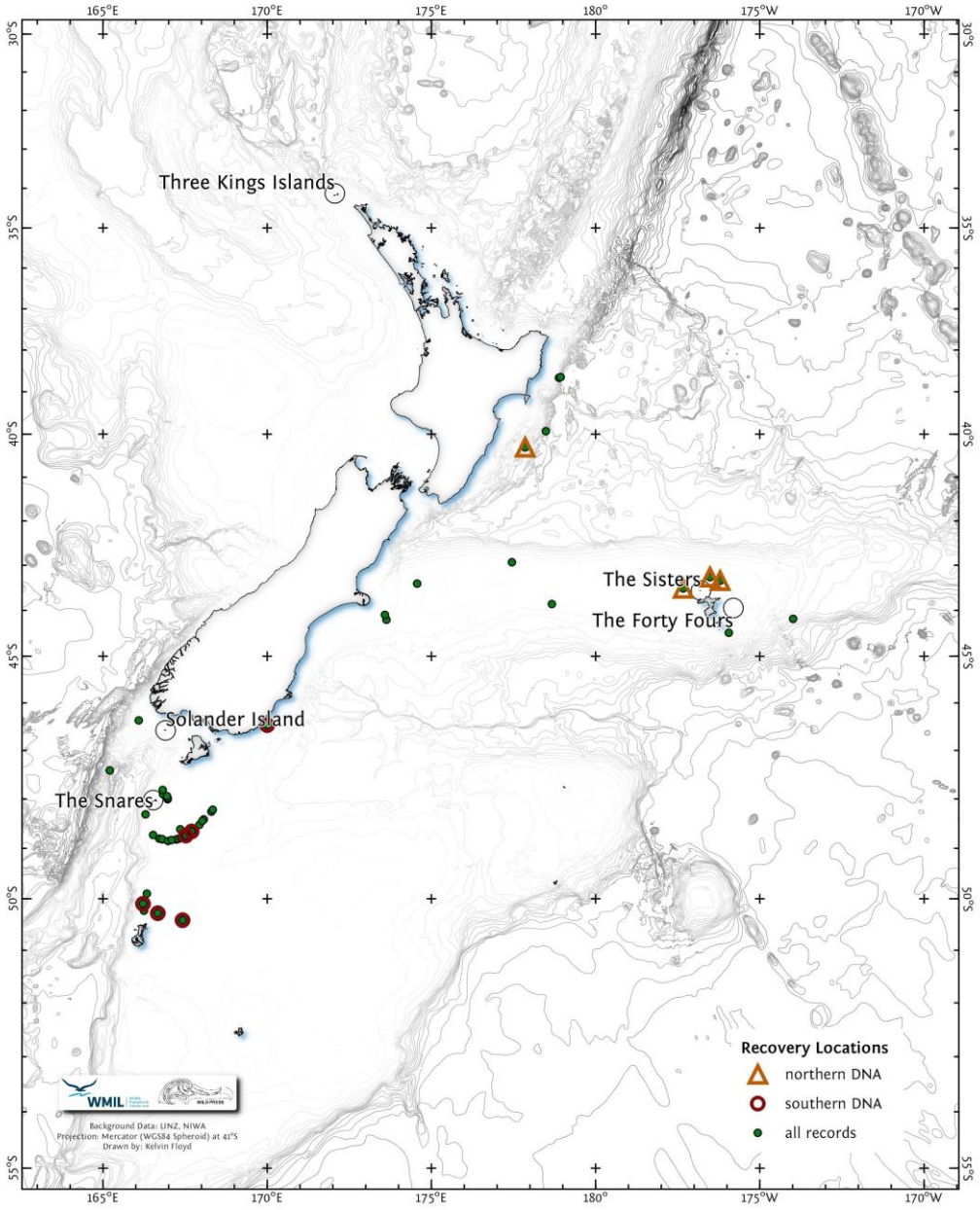
Four period (3 monthly) maps showing the distribution of all Buller's albatross ssp. caught as fisheries bycatch from 4th December 1996 to 30th June 2016 by vessels in New Zealand economic zone, and returned for that period under the annual Conservation Services Programme. Each map also shows the period distribution of birds identified to breeding location by DNA analysis. NOTE. Sampled bycatch bird specimens for DNA analysis were only available from the periods 1996-2005 and 2010-2016. C.J.R. Robertson and E. Bell, using DNA determination analysis by J. Wold, undertook compilation of the datasets for these distribution maps. Kelvin Floyd drew the individual maps.



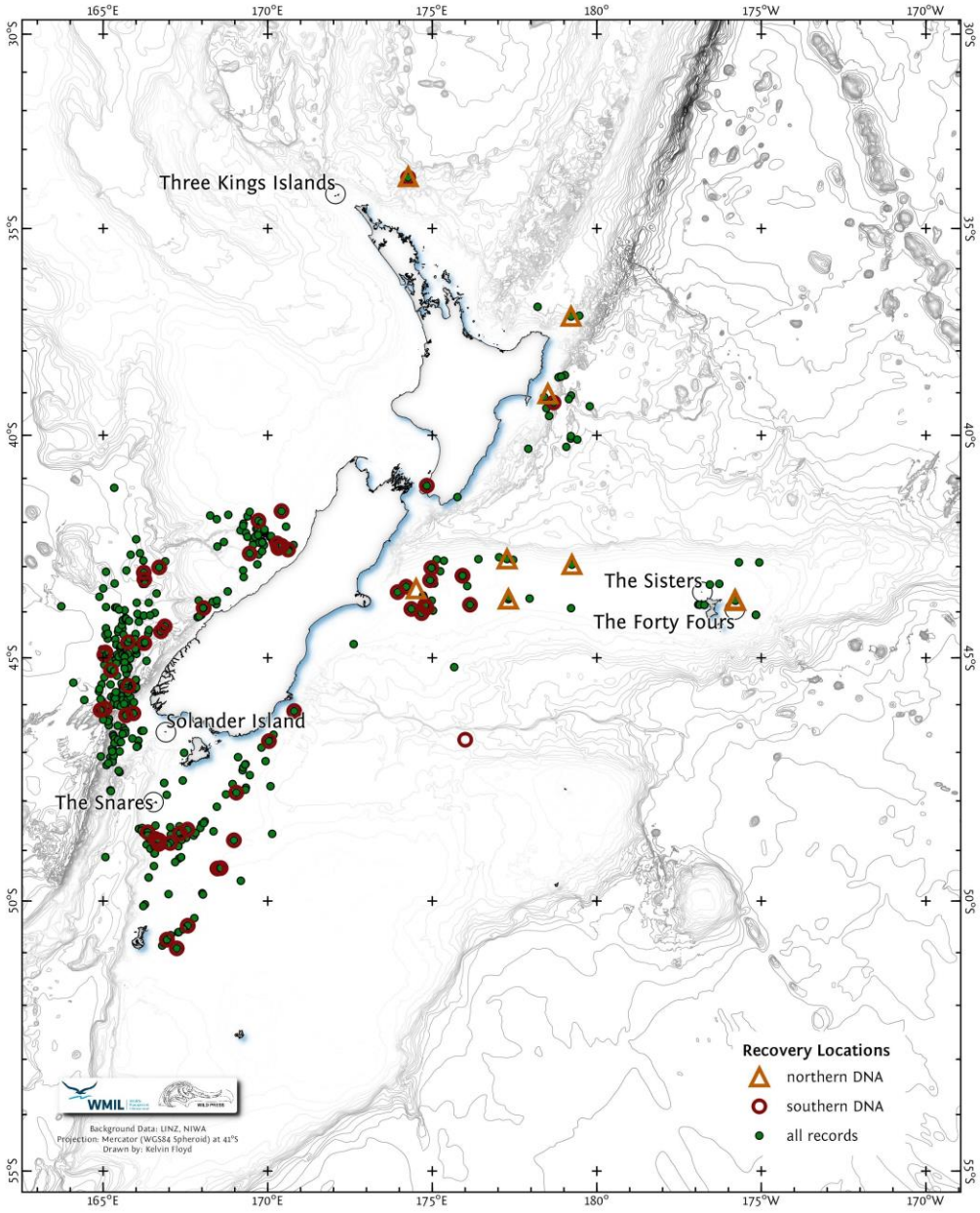
Appendix Figure 5 Quarterly distribution of Buller's Albatross ssp. fisheries bycatch



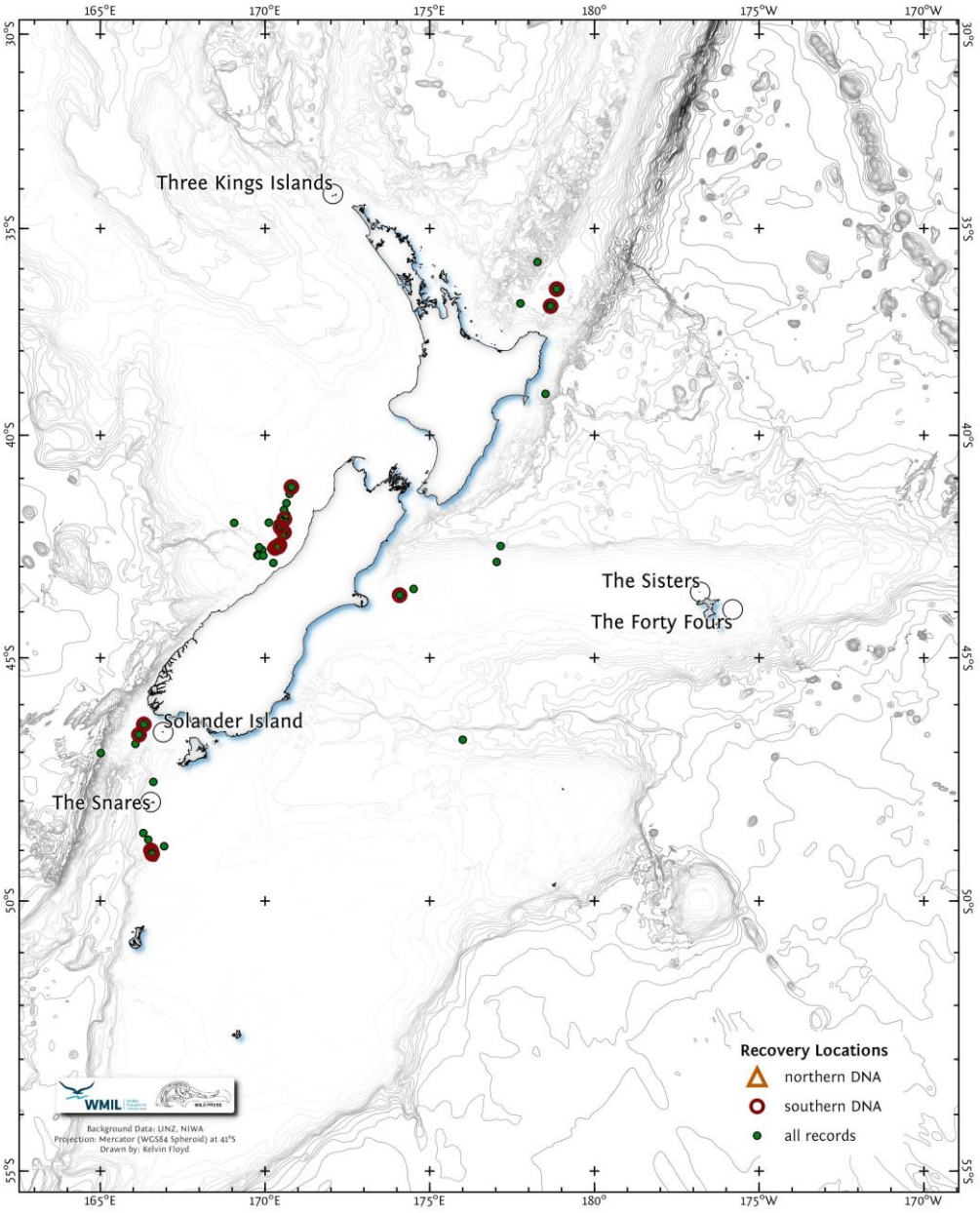
Map a. OCTOBER – DECEMBER. Distribution of 11 Buller's albatross ssp. caught from 4th December 1996 to 30th June 2016. Some 4 birds from this period presented with 'northern' DNA.



Map b. JANUARY – MARCH. Distribution of 51 Buller's albatross ssp. caught from 4th December 1996 to 30th June 2016. Some 4 birds from this period presented with 'northern' DNA and 6 birds presented with 'southern' DNA.



Map c. APRIL – JUNE. Distribution of 468 Buller's albatross ssp. caught from 4th December 1996 to 30th June 2016. Some 9 birds from this period presented with 'northern' DNA and 56 birds presented with 'southern' DNA.



Map d. JULY – SEPTEMBER. Distribution of 60 Buller's albatross ssp. caught from 4th December 1996 to 30th June 2016. Some 16 birds from this period presented with 'southern' DNA.