Conservation genetics of the Threatened Tasman booby (*Sula dactylatra tasmani*)

by

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A thesis submitted in partial fulfilment of the requirements for the
Degree of Master of Science in Zoology
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March 2013

Abstract

Population genetic methods can be employed to inform the conservation of a species in a number of ways. For instance, they can be used to determine if a species has gone through a genetic bottleneck (i.e. a drastic reduction in population size that results in reduced genetic variation), and also if a species exhibits local genetic structure, (i.e., whether there is population genetic structure among neighbouring populations of an otherwise widely distributed species). The objectives of this thesis were to investigate the population genetic structure and long-term effective population size of the recently rediscovered subspecies of the masked booby, the Tasman booby, *Sula dactylatra tasmani*, which unlike masked boobies ,which have a pantropical distribution and are widespread, are range restricted to three island groups in the North Tasman Sea. To achieve this, I apply population genetic methods to mitochondrial control region sequence data, and microsatellite genotype, along with morphometric data. I first examined the cross utility of 43 microsatellite loci developed for the blue-footed *(S. nebouxii*), red-footed (*S. sula*) and Peruvian (*S. variegata)* booby for a population genetic study in my focal subspecies, the Tasman booby. All of these loci amplified in the Tasman booby, and from these 13 independent polymorphic loci were found and used as nuclear data, along with mitochondrial sequence data, to estimate population genetic structure. I also used these two types of data to determine the effective population size of this subspecies, both recently and historically. I found strong population genetic structure from the mitochondrial sequence data, while the microsatellite genotype data revealed weak but significant population genetic structure. I suggest the differences in these two types of marker are most likely due to stochasticity in the mitochondrial genome and/or male-mediated gene flow. Combined, the mitochondrial and microsatellite data revealed the Tasman booby has existed at a relatively stable population size for the last 25,000 years, but estimates of the current effective population size of this subspecies were unreliable. From these combined data I recommend that the Tasman booby should be treated as a single management unit, and conservation efforts from Australia and New Zealand could benefit from communication regarding their management plans. Future work including both autosomal and sex-linked introns could help in resolving the presence or absence of male-mediated gene flow, and/or help estimate an accurate effective population size in this subspecies.

Acknowledgements

Obtaining an MSc degree cannot be done without a tremendous amount of help along the way. There are a large number of wonderful people who deserve mention for all the time, effort and resources contributed to helping me reach submission.

Firstly I must thank my supervisors Tammy Steeves and Marie Hale. Tammy, your enthusiasm and commitment to our monster length meetings has been invaluable. I am indebted to Marie for transforming what appeared at the time convoluted messes into very straightforward solutions on multiple occasions. The two of you have helped me untangle the snares of data analysis and explore so much more about conservation genetics than I could have done on an undergraduate degree alone. I feel privileged to have you as supervisors and am grateful I took the postgrad path.

None of my research would have been conducted without the help of certain people. To Margaret Christian, Emily Favell, Mark Hauber, Richard Holdaway, Stephanie Ismar, Paul O'Neill and Julia Sommerfield, thank you for visiting the islands and collecting the samples required to get me started. Additionally, I would like to thank Julia and Paul for their unpublished morphometric data and Stephanie for providing me with the raw data from her published work to add to my analyses. To the lab technicians Maggie Tisch and Craig Galilee, for arranging my genotyping and sequencing would without a hitch; it is a relief to have one less thing to worry about during lab time. Analysis wise a big thanks to Jamie Morris-Pocock for his help with the somewhat testy LAMARC program, and to Frode Jacobson for aiding me with all the issues I had with BEAUTi and the BEAST.

I would especially like to thank my lab "family". To Jane Andrews, who acted as lab mum and to Andrew Memory for providing the lab with exceptional tunes to get us through the day. I am so fortunate that I got to share my experience with Rachel van Heughten and Ilina Cubrinovska, their continuous optimism, positive attitudes and warmth have made all the difference, and I know we will continue to be friends long after we leave campus.

A good group of friends is always helpful when you are doing a Masters. Thank you to Daniel McRae for proofreading my chapters long after I was sick of them. And thanks to Sophie Head, Aaron Ball, Breanna Sowman, Hayley Clark, Gwendolyn Sugrue and Geoffery Sergeant for their support and all the joy they bring to the table when the going gets tough. I give the sincerest gratitude to my parents Jeannie and Alister and siblings Sarah and Alex for their support and pretending to know what I am rambling about. Finally, I thank my partner Cass Jones, you have a patience and understanding with computer programmes I will never master, and I am lucky to have you in my life.

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Chapter 1 - General Introduction

Introduction

Conservation genetics includes the genetic management of small populations, the resolution of management units and taxonomic uncertainties, and the use of molecular genetics in the understanding of a species' biology (Frankham 2003). It is an applied science, which involves the application of molecular genetics to conservation of biodiversity (see Allendorf and Luikart 2006). The International Union for Conservation of Nature (IUCN) partitions biological diversity into three levels: the gene level, the species level and the ecosystem diversity level (McNeely et al. 1990). Genetics plays a role in all three levels: at the gene level, it is the focus, and at the species level, genetic factors contribute to species extinction risk through inbreeding depression, and genetic structuring of populations which results in a loss of genetic diversity (Frankham 2005, O'Grady et al. 2006). Finally, at the ecosystem diversity level, genetic diversity affects ecosystem survival, diversity and function (Frankham 2010). Gene flow is an evolutionary force that connects populations by moving alleles from one population to another via migrating individuals (Frankham et al. 2010). When gene flow is restricted among small subpopulations, there is less genetic variability available to a species, and it will lose genetic diversity, become inbred and have elevated extinction risks due to such factors as genetic drift, a restricted choice of partners and smaller subpopulation size (Frankham et al. 2010, Saccheri et al. 1998). The Glanville fritillary butterfly (*Melitaea cinxia*) is a well known example of this, where in a highly fragmented metapopulation extinction risk of a population increased significantly with inbreeding. The resulting genetic differentiation between populations with little or no gene flow is known as genetic structure, which can be offset by dispersal (Rousset 2004).

Population genetic methods can be used to address a number of important conservation concerns in a species. For example, they can be used to determine whether a species exhibits local and/or global genetic structure, that is, whether there is genetic structure among neighbouring populations and/or genetic structure among individuals in a population (Karl et al. 1992, Double et al. 2005). Knowledge of genetic structuring is important as it can indicate which populations of a species are in need of management (Keeney et el. 2005, Frankham et al. 2010). For example, a study on shy albatross (*Thalassarche cauta)* and white-capped albatross (*T. steadi*) found high levels of genetic structuring in both species and recommended that each shy albatross population and the three white-capped

populations be treated as separate management units for conservation (Abbott and Double 2003). Multiple studies have estimated genetic structure on a global scale in seabirds (Friesen et al. 2007, Gómez-Díaz et al. 2009, González-Jaramillo and Rocha-Olivares 2011, Steeves et al. 2005a), however, few studies consider structure at the local level (e.g. Reiss et al. 2009, Wallace et al. 2010). Population genetic methods can also be used to determine if populations of a species, or the species itself, has gone through a recent or historical genetic bottleneck in the recent and/or distant past, providing information vital to a species' recovery (Luikart et al. 1998). For example, the alpine ibex (*Capra ibex*) has undergone management via reintroductions, and one population has gone through a bottleneck since its reintroduction, most likely arising from a small number of founders (Maudet et al. 2002).

With recent improvements in genetic technology, researchers need not limit their studies to genetic relationships among populations and species, but can now also address previously unfeasible questions about a species' evolution and ecology. For instance, subjects now available for researchers include: new indicators of structure in multiple seabirds that were once thought improbable (Friesen et al. 2007); rediscovery of species once deemed extinct (Steeves et al. 2010), the joint dispersal patterns of seabirds and their parasites (Gomez-Diaz et al. 2012), why some seabirds exist with little genetic differentiation across large distances while others exhibit highly differentiated populations (e.g. Abbott and Double 2003, Morris-Pocock et al. 2010) and definition of management units for conservation (Palsbøll et al. 2007). Indeed, the progressing field of ecological genetics is allowing researchers to resolve the genetic underpinning of morphological variation in a range of taxa such as ducks (Bulgarella et al. 2012), the pied flycatcher (Lehtonen et al. 2012) and seabirds (e.g. Baião and Parker 2012).

Conservation genetics in seabirds

Numerous genetic studies have assessed several aspects of conservation in seabirds, such as the aforementioned genetic management of small populations, resolution of management units and taxonomic uncertainties, and use of molecular genetics in the understanding of a species' biology (Abbott and Double 2003, Deagle et al. 2007, Quillfeldt et al. 2001). Employment of molecular markers can provide insights to a species' conservation not found when considering ecological data alone. While many seabirds are threatened, the utility of molecular markers can be used to inform

researchers about the conservation status of such species, as non-genetic data alone can be uninformative and/or misleading (see Ludwig et al. 2001).

To be most effective, species conservation must be based on sound taxonomy. If not, failure to recognise reproductively isolated lineages can result in a loss of genetic diversity. Genetic analyses have been crucial to most recent discoveries of new bird species as these are usually "cryptic" birds with inconspicuous or indistinguishable external characteristics resulting from a reliance on nonvisual mating cues, an evolutionarily conservative morphology, or convergent morphological evolution (Bickford et al. 2007). Recent studies have unveiled multiple examples of such cryptic species (e.g. Monteiro's storm petrel *Oceanodroma monteiroi*, Bolton et al. 2008; the long-billed murrelet *Brachyramphus perdix*, Friesen et al. 1996, and the New Zealand storm petrel *Oceanites maorianus*, Robertson et al. 2011).

Additionally, most conservation legislation acknowledges genetically distinct populations of vertebrate species as worthy of protection, for example, "diagnosable units" under the Canadian Species at Risk Act (SARA 2012). The concept is that such populations may be genetically and demographically isolated from each other, and the loss of such populations would result in a loss of some of the species' genetic resources (Frankham et al. 2010).

Evolutionary significant units (ESUs) are used to describe distinct populations containing sufficient genetic diversity to retain evolutionary potential and thus historical population trends along with long term conservation issues (Ashley et al. 2003). In contrast, local populations of a species that are demographically but not genetically isolated from each other may be referred to as management units (MUs) and can be characterised by a variety of tools such as genetic markers, morphology, or life history traits (Ashley et al. 2003). Such populations might employ enough dispersal to prevent genetic differentiation and local adaptation, but it may be an insufficient amount to have the species function as a single demographic unit. Therefore, the degree of connectivity is at a low enough level that each population should be monitored and managed separately (Palsbøll et al. 2007).

An example of determining appropriate MUs is seen in the endemic Hawaiian petrel *Pterodroma sandwichensis*, which was once abundant and now endangered and restricted to four island populations. Using a fragment of the mitochondrial cytochrome *b* gene and nuclear intron sequence data, combined with microsatellite genotype data, Welch et al. (2012) found significant genetic structure among all four populations. From this the authors suggested each island group should be treated as a separate MU and be targeted for conservation actions to prevent any further loss of genetic diversity than what has already resulted from a recent decline in the species. An additional

example can be seen in the yellow-eyed penguin *Megadyptes antipodes,* whose high dispersal suggests that the species functions as a single demographic unit; however differences in the frequencies of the microsatellite alleles and several unique mitochondrial control region haplotypes, combined with assignment tests, indicate that individuals breeding on New Zealand's South Island are demographically isolated from the subantarctic populations, and that the two groups should be treated as separate MUs (Boessenkool et al. 2009).

Estimates of effective population size (or N_e), which is the size of an ideal population that has the same level of genetic drift as the observed population (Fisher 1930, Luikart et al. 2010), are essential to a species' conservation and/or recovery (that is, aiding an already greatly reduced species possibly on the brink of extinction), as it is needed to develop recovery efforts (Mace and Lande 1991, Rieman and Allendorf 2001). It can also be used to predict the extinction risk of a species (Newman and Pilson 1997). For example, this measure has been used in one study involving mitochondrial control region and cytochrome *b* sequence data and microsatellites to estimate the effective population size (among other measures of a species' diversity) in several seabirds located on Christmas Island. Two out of the five species exhibited low effective population sizes, and combined with programs such as BOTTLENECK (Cornuet and Luikart 1996, Piry et al. 1999) and LAMARC (Kuhner 2006), suggested these species had undergone long-term population decline (Morris-Pocock et al. 2012). This finding is extremely helpful for the conservation of these species, as it can help set conservation priorities. Estimates of N_e may also be useful to determine if a small population has always been small (i.e., has not experienced a significant decline in numbers in the recent and/or distant past). Knowledge that a population is naturally small can be helpful as it would suggest that immediate conservation management action to increase population size is not required.

Many studies have already been conducted concerning the genetic structure of seabirds. For example, Levin and Parker (2012) characterised genetic differentiation in the Nazca booby (*Sula granti*) and great frigatebirds (*Fregata minor*) by analysing eight microsatellite loci and three mitochondrial genes (cytochrome *b*, NADH dehydrogenase subunit 2 and cytochrome oxidase I). The Nazca boobies were genetically differentiated across the Galapagos archipelago into three distinct groups, while the great frigatebird had little to no significant genetic differentiation among populations. Burg and Croxall (2004) investigated global structure in four species of the wandering albatross (*Diomedea antipodensis*, *D. exulans D. gibsoni and D. dabbenena*). Researchers found three of the species to be genetically differentiated; however, *D. exulans* did not express any genetic differentiation despite widespread distribution (Burg and Croxall 2004). Excluding this thesis and the above Levin and Parker (2012) study, very few studies have examined genetic structure at a local

level in seabirds. In contrast, numerous studies concerning global structure have been published (Abbott and Double 2003; Burg and Croxall 2001; Friesen et al. 2007; Gómez-Diaz et al. 2009).

However, several studies concerning global genetic structure suggest that local structure might exist in these seabird species, which is relevant to seabird conservation as some species may need to be monitored at the local level (Steeves et al. 2005a, 2010, Morris-Pocock et al. 2010b).

The Tasman booby

The Tasman booby (*Sula dactyaltra tasmani*) is a recently rediscovered subspecies of the masked booby, *S. dactylatra*. Masked boobies are large plunge-diving seabirds that breed on oceanic islands in a pantropical distribution and display high natal philopatry (Steeves et al. 2005b, Steeves et al. 2010). The Tasman booby differ from other subspecies of the masked booby in a number of physical traits; firstly, they have sepia eyes unlike their yellow eyed sister taxa, which was first discovered in O'Brien and Davies 1990 from examination of museum skins, and later confirmed by Ismar et al. (2010) using live birds. Secondly, *S d. tasmani* have larger wing spans than both the *S. d. personata* and *S. d. bedouti* subspecies (O'Brien and Davies 1990). However, limited data is available concerning physical differences within this subspecies, which is limited to three island groups: Lord Howe Island, Norfolk Island and the Kermadec Islands (the Kermadecs from herein) (Steeves et al. 2010). One study has gathered some measurement data, however the individuals used were from one island group only (Ismar et al. 2010). Additional morphometric data could be informative, as differences among island groups may be indicative of underlying gene flow restrictions (Dearborn et al. 2003, Steeves et al. 2005b).

While the masked booby is listed as a species of least concern (IUCN 2012), the Tasman booby is listed as nationally endangered in New Zealand (Miskelly et al. 2008) and nationally vulnerable in Australia (Garnett and Crowley 2000). Relative to the other three subspecies of masked booby, the Tasman booby has the lowest number of breeding pairs, with estimates of around 100 breeding pairs on the Kermadecs, around 350 on Norfolk Island and less than 500 on Lord Howe Island (Priddel et al. 2005). Previous studies have been conducted on masked boobies at the global scale, for example, mitochondrial sequence data suggests that there is significant global population genetic structure (Steeves et al. 2003, 2005a, b) and there is also some evidence of genetic structure in the Tasman booby (Steeves et al. 2010). However, Steeves et al. (2010) included a limited number of samples from Lord Howe Island (n=30), Norfolk Island (n=11) and the Kermadecs (n=14). So, overall,

while there is data concerning genetic structure on a global scale (i.e., for the species), limited research has been conducted at a local scale (i.e., for subspecies with a restricted geographic distribution like the Tasman booby).

Little is known about the size history or current conservation status of the Tasman booby. Approximately 3,163 nestlings were banded in the Norfolk Island group from 1981-2007 (Priddel et al. 2005). From the banding data there has been evidence of movement from nestlings banded on Lord Howe to the Kermadecs (O'Brien and Davies 1990), and movement of an adult from Norfolk island to the Kermadecs (O'Brien and Davies 1990), but there are no records of dispersal as of yet (i.e., a chick banded in one island group found breeding in another island group). While previous research has shown that these birds are genetically different from birds elsewhere in the Pacific (Steeves et al. 2005b, 2010) and that birds nesting on the three main island groups within the North Tasman Sea may be genetically different (Steeves et al. 2010), these studies had limited sampling and data was restricted to mitochondrial control region sequence data. The addition of microsatellite markers can reveal more recent changes at the population level, so a more extensive study with additional samples and genetic markers, as well as morphological data, could aid the conservation of this species immensely.

Molecular Genetic Methods

Information about the evolutionary history of a species is maintained in its DNA. Several methods have been developed to assay DNA information either directly or indirectly, and many problems previously associated with these molecular genetic tools are gradually being resolved (see Hudson 2008). Until recently, molecular genetic tools were costly to adapt to novel research projects and slow to develop, and assembling enough variable markers for new focal species was also a challenge. However, increasingly sophisticated DNA-based technologies, including high-throughput sequencing, are enabling researchers to develop informative molecular genetic tools in an efficient and cost effective way (e.g. Abdelkrim et al. 2009, Allentoft et al. 2009).

One of the first methods employed was DNA fingerprinting which uses restriction fragments to test individuals for variation in a number of repeats in a 20 to 25bp unit (Krawczack and Schmidtke 1998). However, such methods of mutation detection have been mostly supplanted by tools based on DNA amplification via polymerase chain reaction, or PCR, which looks at an individual's alleles. A common technique of PCR is using microsatellite analysis, which applies specially designed PCR primer pairs to

amplify gene regions that vary in the number of short, tandem repeats (typically two to six bps). Microsatellites are biparentally inherited markers with a high mutation rate located at high frequency in the nuclear genome of both prokaryotes and eukaryotes (Kupper et al. 2008). The tandem repeats are created from slippage in DNA replication and recombination (Li Y. C. et al. 2002). Microsatellites are now being used to determine a number of aspects about a species' conservation such as species identification (Scribner and Bowman 1998), individual assignment (Primmer et al. 2000) and recent bottleneck detection (Spencer et al. 2000).

Mitchondrial DNA (mtDNA) sequences may also be used to inform conservation. MtDNA is haploid and predominately uniparently inherited from the maternal line (but see White et al. 2008 regarding paternal leakage), with a much lower mutation rate than microsatellites, possibly by a factor of 10 (Vawter and Brown 1986). MtDNA can be used to inform researchers about historical population size, female mediated gene flow, and genetic structure (Ballard and Whitlock 2004, Sunnucks 2000). The mitochondrial genome has both coding and noncoding genes that differ in their mutation rates (Lemire 2005); for example, cytochrome *b* is a protein-coding gene with a relatively slow mutation rate that has been used in numerous phylogenetic and phylogeographic studies, whereas the control region is a non-coding region with a relatively fast mutation rate that has been used in numerous phylogeographic and population genetic studies (Baker 2000).

For example, most boobies in the Pacific Ocean including red-footed (*Sula sula*), brown (*S. leucogaster*) and masked (*S. dactylatra*) have a single cytochrome *b* haplotype (Steeves et al. 2003), whereas dozens of control region haplotypes exist at varying frequencies (Morris-Pocock et al. 2010a, Steeves et al. 2005a). Caution is advised when using control region sequences, however, as nuclear homologues of the control region (e.g. Kidd and Friesen 1998), and duplicated control regions (e.g. Eberhard et al. 2001; Abbott et al. 2005 and Morris-Pocock et al. 2010a) have been observed in many bird species.

New approaches to analyses of the data are also improving the advancement of the field of conservation genetics. For instance, the program STRUCTURE can be used to estimate the populations present without *a priori* knowledge (Evanno et al. 2005), which is a deviation from traditional population genetics like F_{ST} that require a set of determined populations *a priori*. For example, Nomura et al. (2012) used STURCTURE to determine the number of population clusters in East Asian indigenous goats, and found they should be grouped into the following three genetic clusters: East Asian, Southeast Asian and Mongolian. In addition, STRUCTURE can also be employed to detect migrants (e.g. Morris- Pocock et al. 2011) and assign unknown individuals to a source (e.g. Parker et al. 2004). The program LAMARC has been created to estimate historical demographics in populations

by acting as a Markov chain Monte Carlo (MCMC) genealogy sampler (Kuhner 2006). Additionally, the program BEAST can reconstruct a species' phylogeny and format Bayesian skyline plots (Drummond et al. 2005). A Bayesian skyline plot also uses a Bayesian approach to estimate a posterior distribution of effective population size (N_e) through time directly from a sample of gene sequences, under any nucleotide-substitution model (Drummond et al. 2005). For example, Morris-Pocock et al. (2012) used LAMARC for the dual purpose of estimating the population mutation rate and the rate of population growth for both Christmas Island frigatebirds (*Fregata andrewsi*) and Abbott's boobies (*Papasula abbotti*).

DNA based studies of a species' conservation work best if combined with multiple markers, as these markers can inform researchers about a species' recent conservation status, as well as any historical changes in population size which could ultimately be responsible for a loss of genetic diversity. For example, Dickerson et al. (2010) used both microsatellites and control region mtDNA sequence data to test for population structure in the Northern fur seal (*Callorhinus urs*) and concluded from their data that the absence of genetic structure found was likely due to insufficient time since rapid population expansion events combined with low levels of contemporary migration. Additionally, Morris-Pocock et al. (2008) used mitochondrial control region, microsatellite and intron loci to investigate global genetic structure in common murres (*Uria aalge*). They found that both Pacific and Atlantic populations diverged during the Pleistocene and were not exchanging migrants. Furthermore, while little genetic structure was found within the Pacific populations, significant east-west structuring was found among Atlantic colonies (Morris-Pocock et al. 2008).

Objectives of thesis

The ultimate goal of this thesis is to investigate the conservation genetics of the Tasman booby. To achieve this, I apply population genetic methods to mitochondrial control region sequence data, and microsatellite genotype data to examine the genetic structure, and long-term effective population size of the Tasman booby.

In Chapter 2, I explore the utility of microsatellite loci developed for other booby species. Using 43 microsatellite loci previously developed for a number of its congeners, I examine the amplification and polymorphism in the Tasman booby. From this data, I screen all polymorphic loci against three avian genomes to determine if these loci are independent. From this assessment, I use the loci that are independent and polymorphic in the Tasman booby, along with control region sequence data

and morphological data, to answer questions concerning the conservation of the Tasman booby in Chapter 3. I estimate the extent of local genetic structure using both mitochondrial control region sequences and nuclear microsatellite genotypes. I also estimate the effective population size of the Tasman booby and determine whether it has changed over time. These data are combined with new morphometric data to determine the appropriate number of management units to assist the management of this subspecies. For example, if the Tasman booby is functioning as a single panmictic management unit, conservation efforts would need to run simultaneously, while if the Tasman booby is separated into two or three management units, each management unit can be monitored at different time frames.

Given that little is known about the conservation status of the Tasman booby, knowledge of the local genetic structure of this subspecies, whether it has gone through a relatively recent or more distant decline in effective population size, and any evidence of morphological variation between individuals would be a strong step forward towards aiding their conservation. The discovery of any cross species utility with other booby microsatellite markers will also be of considerable help as they will eliminate the effort and cost required to process species-specific primers; these can also be used by other researchers looking to study these birds.

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Chapter 2 – Diversity and utility of microsatellites from non focal species

Introduction

Population genetics concerns the study of allele frequencies under the influence of the four main evolutionary process of natural selection, mutation, gene flow and genetic drift (Wright 1984). Understanding the population genetics of a species, such as the recently rediscovered Tasman booby *Sula dactylatra tasmani*, can help researchers determine if a species has gone through a genetic bottleneck, inbreeding depression, or if there is genetic structuring between island populations; these factors can ultimately lead to extinction in a species (Frankham 1995, 1998). To gain a better understanding of the population genetics of a species, the use of multiple, effective markers is fundamental.

Early population genetics studies used allozymes to determine aspects such as linkage disequilibrium (Langley et al. 1974) and amino acid polymorphism (Verrelli and Eanes 2000), but the field has since expanded to include a wide array of genetic markers to assay genetic variation within and among populations. Such markers include RFLPs (Botstein et al. 1980), AFLPs (Moghaddam et al. 2005), SNPs (Sachidanandam et al. 2001) and microsatellites (Bowcock et al. 1994). Of these markers, microsatellites have emerged as arguably the most useful marker for determining processes occurring in a species or population (Helyar et al. 2011, Selkoe and Toonen 2006).

Microsatellites are short tandem repeats of 1-6 nucleotides located at high frequency in the nuclear genome of most prokaryotes and eukaryotes (Kupper et al. 2008). They can also be referred to as short tandem repeats (STR), simple sequence repeats (SSR), and variable number tandem repeats (VNTR) (Selkoe and Toonen 2006). A microsatellite locus will typically vary in length between five and 40 repeats. The chief mutational processes responsible for such variability are deemed to be replication slippage and recombination (Li et al. 2002). Both of these processes alter the length of the microsatellite by changing the number of repeats present (Li et al. 2002). Microsatellites appear to be nonrandomly distributed across the genome, with a large proportion of microsatellites located in non-coding sequences; which follows with that the majority of the genome is non-coding (Selkoe and Toonen 2006). Microsatellites are also considered to be selectively neutral (Selkoe and Toonen 2006). However some microsatellite loci are located in functional regions involved in regulation of gene activity, DNA replication and recombination, and chromatin organisation (Li et al. 2002). Both

trinucleotide and hexanucleotide repeats are most likely to appear in protein coding exons of all taxa as they do not cause a frameshift (Tóth et al. 2000).

While using these regions as molecular markers was originally developed for use in the human genome (Weber and May 1989), microsatellites are now implemented in myriad species for a range of uses such as determining paternity (Knight et al. 1998) individual assignment (Primmer et al. 2000) and species identification (Scribner and Bowman 1998). The utility of microsatellites spans the animal, plant and fungi kingdoms (Abdelkrim et al. 2007, Craft et al. 2007, Lim et al. 2004). Dinucleotide, trinucleotide and tetranucleotide repeats are the most common choices in genetic studies (Kupper et al. 2008) as dinucleotide repeats can account for the majority of microsatellites in many species (Li Y. C. et al. 2002), and trinucleotide repeats are most likely to appear in coding regions as they do not cause a frameshift (Tóth et al. 2000). Because they are bi-parentally inherited with a high mutation rate and are presumed to be independent, microsatellites are highly appropriate for investigating genetic questions such as structure, effective size of a population, or the recent size increase or decline at a population level.

When investigating genetics within and among populations, microsatellites often need to be developed for the focal species if there is not already an extensive library available. Traditionally, microsatellite loci have been developed via cloning methods, which entails isolating loci from a partial genomic library of the target species (Zane et al. 2002): high quality genomic DNA is reduced to fragments by either restriction enzymes or by sonication. The selected fragments are then ligated into a common plasmid vector either after ligation of directly to specific adaptors. The transformation of ligation product with bacterial cells can usually yield thousands of recombinant clones; these can then be screened for the presence of microsatellite sequences. Screening is generally carried out via Southern hybridisation using repeat-containing probes. Repeat containing clones are identified and sequenced, then specific primers are designed and PCR conditions are optimised to provide the amplification of each new locus for different individuals of a population. This method, however, is very costly and can take months to complete (Zane et al. 2002).

To avoid this, researchers have recently turned to ‘next generation’ sequencing technologies to generate species specific microsatellite libraries. For example, the 454 sequencing method conducted using a Genome Sequencer FLX (GS-FLX) System (Roche, Penzburg, Germany) at a $1/16^{th}$ scale produces tens of thousands of reads ranging from 200 to 300 bp in length (Abdelkrim et al. 2009, Allentoft et al. 2009).

A full run on the GS-FLX System using an LR70 plate usually produces more than 400,000 reads and might be expected to produce 100,000 microsatellite sequences (Abdelkrim et al. 2009). If scaled down to the 1/16th format, you might expect to isolate 6-7,000 microsatellites in a single run (Abdelkrim et al. 2009). A large proportion of these microsatellites will be discarded by the end user (e.g., many will be too close to the end of the fragment to enable design of flanking PCR primers), resulting in around a few hundred microsatellites of potential use to be considered as markers. Only reads that have large, pure repeats will be selected, reducing the number of useable loci to 10-100. 454 sequencing can cut the cost of microsatellite development by 3-5 times (Abdelkrim et al. 2009) and can be drastically quicker than the cloning method. In light of this new technique, we should expect to see an increase in the amount of species specific microsatellite loci available.

Regardless of how microsatellites are generated, the utility of these loci may not be strictly limited to the species they were designed for, and researchers initiating work on a species that has not been studied previously are advised to check which primers are available first before delving into species specific primer development. Cross species amplification is predominately trialled against closely related taxa, with success found in plants (Gonzalez-Martinez et al. 2004) mammals Griffin et al. 2001) and fish (Williamson et al. 2002). However, this practice appears to be most widely implemented in birds (Galbusera et al. 2000, Huang et al. 2005, Kayang et al. 2002). Many studies have successfully used microsatellites for cross-species amplification including parrots (Taylor 2011), shorebirds (Kupper et al. 2008, Williams et al. 2012), pheasants (Mukesh et al. 2011) and passerines (Garcia-Vigon et al. 2008). An assortment of variables can affect the cross amplification and polymorphism in avian species, including the analysis conditions and the molecular characteristics (such as the number of repeat units observed) of the source loci (Primmer et al. 2005). First, primers developed for closely related species are more likely to be polymorphic (Primmer et al. 2005, Taylor 2011). For example, Primmer et al. (2005) found in their study of 32 bird species that the proportion of polymorphic loci that amplified in other species decreased with increasing phylogenetic distance. Second, from pooling information from previous studies containing a total of 331 loci from 32 avian species, Primmer et al. (2005) found that factors such as a high number of repeat units in the source species resulted in a higher probability of amplification/polymorphism success in the non-source species. In general, Barbarà et al. (2007) found from a compilation of cross-species marker transfer studies ranging from animals to plants to fungi, that the microsatellites that amplified best resided in taxa which had long generation times, mixed or outcrossing breeding systems, and a small genome size in the target species compared to the source species the marker was developed for.

Regardless of whether researchers use microsatellite loci developed for focal species or not, our knowledge of avian genomes is expanding, to the extent that these once anonymous markers are beginning to shed some of their anonymity. In birds, cytogenetic studies first revealed that the ancestral avian karyotype is markedly similar to the chicken karyotype (Griffin et al. 2001). The avian genome has, on average, 40 pairs of chromosomes (Griffin et al. 2007). One of these pairs are the sex chromosomes, labelled Z and W. The remaining chromosomes are known as autosomes. In birds, the females are the heterogametic sex, having a karyotype of ZW while males are homogametic with a karyotype of ZZ (Daniel et al. 2007).

Synteny (when the same loci are physically located on the same chromosome for two or more species and can also be reported as a lack of interchromosomal rearrangement) appears to be common in avian genomes. Dawson et al. (2007) found synteny between a chicken microsatellite map and a great reed warbler linkage map, in several autosomes and the Z chromosome. Backström et al. (2006) provide additional evidence for high synteny: 23 genes spread over the Z chromosome of the chicken genome were also all located on the Z chromosome in the collared flycatcher. Further research compiled a linkage map of the collared flycatcher comprising 33 autosomes, all presenting high synteny with only two interchromosomal rearrangements occurring with the chicken genome, despite the lineages separating an estimated 100 million years ago (Mya) (Backstrom et al. 2008). Hale et al. (2008) constructed genetic linkage maps of the homologue of chicken chromosome 7 for the zebra finch and house sparrow from a combination of microsatellites and SNPs and found synteny was well conserved within the chromosome.

Synteny is not to be confused with gene order. Gene order simply means the location of a locus relative to other loci on a chromosome. When gene order is conserved it can also be reported as a lack of an intrachromsomal rearrangement. Gene order appears to be better conserved in bird genomes of closely related species. For example, Backström et al. (2008) compiled a linkage map of the collared flycatcher from the chicken genome. The authors found that despite diverging 100 million years ago, only a few intrachromosomal rearrangements had occurred. Backström et al. (2010) developed Z chromosome maps of both the collared flycatcher and the pied flycatcher to test if chromosome rearrangements accounted for the post-zygotic reproductive isolation between the two species. When no evidence for chromosomal rearrangements was found, the authors concluded that their study did not provide support for such a hypothesis. However, one must remember that these are closely related species that have a short evolutionary divergence time; more distantly relate species may have less similar gene order. For instance, Itoh et al. (2006) located 14 zebra finch genes which are present in the Z chromosome in chickens. While all genes were located on the Z

chromosome in the zebra finch (i.e. there was evidence for high synteny), the gene order on the zebra finch Z chromosome was strikingly different to its chicken counterpart. The authors hypothesised that up to four inversions were required to create such an altercation in gene order between the lineages. Additionally, when comparing linkage maps of the great reed warbler and the collared flycatcher with the chicken genome, Hale et al. (2008) found gene order rearrangements in autosomes between the passerines and chicken. The Itoh et al. (2006) and Hale et al. (2008) studies suggest genomic instability at the level of gene order and organisation across distantly related taxa of birds.

With advances in genome sequencing, more data is emerging for comparison. The sequencing of three bird genomes, the chicken (*Gallus gallus)* the zebra finch (*Taeniopygia guttata)* and the turkey (*Meleagris gallopavo*) has highlighted well conserved synteny in the avian genome, with relatively few large-scale interchromosomal rearrangements detected (Derjusheva et al. 2004, Warren et al. 2010). This synteny exists between species in distantly related orders (Backstrom et al. 2008) and even between widely diverged species such as turtles and birds (Matsuda et al. 2005). The sequenced genomes of the chicken, zebra finch and turkey can also be used as a comparison when mapping microsatellite loci for a specific bird genome; as these genomes are completely sequenced and bird genomes have high synteny of microsatellite sequences, mapping loci in question on other, known bird genomes can help indicate where a locus might be found in other bird genomes (Backstrom et al. 2006, Backstrom et al. 2008, Reed et al. 1999).

High synteny suggests we can now map loci to a specific chromosome with a reasonable level of confidence in birds. Loci developed for specific birds can be assigned a location on a chromosome by utilising sequence homology with avian genomes. For example, Dawson et al. (2012) used a compilation of already existing house sparrow microsatellites and loci developed from other passerine species to assign predicted locations in the house sparrow genome. 134 loci were assigned to 25 different autosomes and 8 loci to the Z sex chromosome based on the zebra finch genome. However it should be emphasised that the assigned locations are predictions, as they are based on the zebra finch genome and small-scale rearrangements are to be expected between these two closely related species (Dawson et al. 2012). Regardless, from these findings, researchers will now have more evidence when checking whether loci are physically linked or not.

However, as discussed above (Backstrom et al. 2008, Backstrom et al.2010, Hale et al. 2008, Itoh et al. 2006) changes in gene order also means that while a locus may be on the same chromosome across different genomes, it may not be in the exact same location on the chromosome, and researchers may not have the confidence to state where they are in relation to other loci (Hale et al.

2008). Therefore, estimates of gene order should be made with caution or not at all. Despite this, this deluge of sequence information is helping to take away the previous anonymity of microsatellites and can inform researchers if they are dealing with independent loci. For a locus to be independent, they must not be genetically linked (that is, inherited together more often than expected under random inheritance). Microsatellites can be physically linked by being located on the same chromosome, but may not be genetically linked if they are located far enough apart on the chromosome to not be inherited together. It is important to determine the independence of a microsatellite intended for population genetics research – a linked locus, if used in population structure analyses unchecked, can result in providing statistical power to a study that in reality is not there.

The Tasman booby (*Sula dactylatra tasmani*) makes an excellent study species for a population structure study using microsatellites. As a recently rediscovered subspecies of the masked booby (*S. dactylatra*, Steeves et al. 2010), little is known about the size history or current conservation status of this species. An understanding of structure in this species can help indicate if the Tasman booby is a large genetically stable population, or a group of more isolated populations only breeding with individuals in the same island group. Knowledge of effective population size would also be of immeasurable help as it can indicate a species' risk of extinction (Luikart et al. 2010). Previous research based on mitochondrial control region sequence data shows that these birds are genetically different from birds elsewhere in the Pacific (Steeves et al. 2005b, 2010) and that birds nesting on the three main island groups within the North Tasman Sea may be genetically different (Steeves et al. 2010). However, mitochondrial DNA is uniparently inherited (Ballard and Whitlock 2004) in contrast to the biparently inherited microsatellites. Additionally, relative to mitochondrial control region sequences, microsatellites have higher resolution due to their rapid evolution (Li et al. 2002), which can show us recent changes at a population level. Microsatellites have not been developed for the Tasman booby, but 43 microsatellite loci already exist for several of its congeners (Faircloth et al. 2009, Morris-Pocock et al. 2010, Taylor et al. 2010b). In this chapter, I investigate the utility of microsatellite loci developed for the blue-footed *(S. nebouxii*), red-footed (*S. sula*) and Peruvian (*S. variegata)* booby for a population genetic study in my focal subspecies, the Tasman booby. In particular, I address the following question:

Will cross-amplification of 43 loci developed in other booby species yield at least 12 independent polymorphic loci in the Tasman booby?

Materials and methods

Sample collection and DNA extraction

Blood samples were collected from 86 individuals at different stages of development (chicks, juveniles, adults, see Appendix I) including two samples that were collected from the same individual to serve as a blind test (see Appendix I). 27 samples were collected from Norfolk and Philip Island (Norfolk Island Group), 30 from Lord Howe Island (Lord Howe Island Group) and 16 from the Kermadecs (Kermadecs Island Group) in the Tasman Sea. Blood was stored either on filter paper or in lysis buffer. DNA was extracted using either a Chelex protocol (as per Hillis 1996) or an Invitrogen PureLink Genomic DNA Kit. As bird blood has nucleated erythrocytes, I used the mammalian tissue protocol as per the supplier's recommendations.

Sexing by PCR analysis

Sexing data was in hand for 21 samples based on previous sexing analyses (Ismar et al., *unpublished data*) and vocalisations (J. Sommerfield, *unpublished data*). For the remaining samples, sexing PCRs were performed using an Eppendorf AG 22331 Hamburg thermal cycler in 16µL volumes comprising 1x NH_4 buffer, 3mM $MgCl_2$, 0.4U Taq, 1 µL genomic DNA, 0.4µM of each primer, 200µM of each forward 2550F (5'-GTTACTGATTCGTCTACGAGA-3', (Fridolfsson and Ellegren 1999)) and reverse 2718R (5'-ATTGAAATGATCCAGTGCTTG-3', (Fridolfsson and Ellegren 1999) primer and ddH_2O to the final volume. Thermal cycling conditions followed a touchdown protocol as follows: 2 min at 94°C, followed by cycles of 95°C for 30 s, annealing initially at 60°C for 30 s, and extension at 72°C for 30 s. Each cycle decreased in annealing temperature by 1°C until reaching an annealing temperature of 50°C, where 30 cycles were run in this condition. A final extension completed the reaction at 72°C for 5 min. PCR products were electrophoresed through a 3% agarose gel stained with Sybersafe and visualised under ultraviolet light to verify product band size. Individuals were determined female by the presence of two bands; males were determined by the presence of one band in at least two separate PCRs.

Microsatellite analysis

Samples were tested for amplification at 43 microsatellite loci originally developed for Peruvian (Taylor et al. 2010b) blue-footed (Faircloth et al. 2009, Taylor et al. 2010b) and red-footed (Morris-Pocock et al. 2010b) boobies. All PCR reactions were performed using an Eppendorf AG 22331 Hamburg thermal cycler in 16µL reaction volumes containing approximately 1x NH_4 buffer 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer, 0.4 U Taq and 1µL DNA. Thermal cycling conditions followed a touchdown protocol as follows:

Samples were denatured at 95°C for 2 min, followed by an initial cycle of 95°C for 30 s, annealing at 60°C for 45 s and 72°C for 30 s. Subsequent cycles would have a reduced annealing temperature by 0.5°C until reaching an annealing temperature of 52°C. 20 cycles would follow with this annealing temperature with a final extension of 72°C for 2 min. Following amplification PCR products were visualised on a 1% agarose gel stained with sybersafe. Samples were then tested for polymorphism, using at least two individuals from each of the three population groups. Samples were tested for polymorphism using the same 16µL reactions above except that the F primer concentration was reduced to 0.13µM to compensate for the addition of a fluorescently tagged primer at a concentration of 0.5 µM. The F primer was also tagged with an M13 primer which had a 6-FAM fluorescent label. The same touchdown protocol was followed, excluding the two loci (BOOB-RM4-C03 and BOOB-RM4-G03) where the touchdown range was extended to a final annealing temperature of 50°C. Sizes of loci were determined by co-running a size standard (Genescan™-500 LIZ™ and Genescan™-1000 ROX; Applied Biosystems, Melbourne) on the sequencer Applied Biosystems, 3130xl Genetic Analyze; fragments were then scored manually with the use of GeneMarker v1.8 Software (Soft Genetics).

Data analysis

Sex linkage

The genotype data for all polymorphic loci was divided into males and females to look for evidence of sex linkage. If a locus yielded only homozygotes in the female samples, it was a candidate for sex linkage.

General diversity indices

The mean level of observed (*H*o) and expected (*H*E) heterozygosity, and mean number of observed (*Na*) and effective (*ne*) alleles were estimated using GenAlEx v6.41 (Peakall and Smouse 2006). To test for significant linkage disequilibrium and to test for significant deviations from Hardy-Weinberg equilibrium (HWE), ARLEQUIN v3.5 (Excoffier and Lischer 2010) was used. Both tests were corrected for multiples comparisons (Narum 2006).

Chromosome location

Microsatellite clonal sequences of polymorphic loci were compared against the chicken (*Gallus gallus* Build 3.1), turkey (*Meleagris gallopavo,* Build 1.1) and zebra finch (*Taeniopygia guttata,* Build 1.1) genomes in BLAST v2.2.4 (Altschul et al. 1998) using a web based NCBI nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/). The default parameters for three search algorithms were used: megablast, discontiguous megablast and blastn. Although discontiguous megablast is intended for cross-species comparisons, preliminary analyses indicated that additional loci could be located using megablast or blastn. Chromosomal locations were determined for loci with BLAST scores over 70 bp in length, with an E-value of 1e-05 or lower, and were at least 1e-05 or lower than their second best hit (Dawson et al. 2007). A predicted microsatellite map was produced using MAPCHART software (Voorrips 2002).

Results

Microsatellite analysis

All 43 microsatellite markers developed for other booby species amplified successfully. However, amplification came at varying levels of success: while some gave clear, single bands after the first amplification, other loci produced multiple bands and/or a DNA "smear" in the gel. Both a smear and amplification of multiple bands occurred in loci BOOB-RM4-C03 and BOOB-RM4-G03. The PCR conditions were altered in their volumes of $MgCl_2$, ranging from 2.5-1.5 Mm. A volume of 2.0 mM appeared to reduce the extra bands and smear most. An altered touchdown protocol was also implemented for these loci, such that the touchdown range was extended to a final annealing temperature of 50°C.

Sixteen of the 43 loci were polymorphic based on at least two individuals from each island group. One of these loci was found to be sex linked: as it was homozygous in all females, and was found to be located on the Z chromosome in all three avian genomes (Sn2A-36, see details below). Another locus appeared to amplify two loci simultaneously (Ss1b-99) so both were discarded from downstream analyses (see Table 1).

Table 1. List of loci assessed for the amplification and polymorphism in the Tasman booby. Outcomes when cross amplified with the Tasman booby *Sula dactylatra tasmani*

Locus Monomorphic	Locus polymorphic	Locus amplifying more than one area	Locus sex linked
BOOB-RM3-D07	BOOB-RM2-F07	Ss1b-99	Sn2A-36
BOOB-RM4-A08	BOOB-RM3-F11		
BOOB-RM4-B03	BOOB-RM4-C03		
BOOB-RM4-E10	BOOB-RM4-D07		
Sn2A-90	BOOB-RM4-E03		
Sn2A-123	BOOB-RM4-F11		
Sn2B-68	BOOB-RM4-G03		
Sn2B-83	Sn2B-83		
Ss1b-16	Sn2B-100		
Ss1b-51	Ss2b-71		
Ss1b-57	Ss2b-138		
Ss1b-88	Sv2A-26		

Ss1b-98 Ss1b-99 Ss1b-106 Ss1b-142 Ss2b-2 Ss2b-35 Ss2b-48 Ss2b-88 Ss2b-92 Ss2b-110 Ss2b-153 Sv2A-2 Sv2A-47 Sv2A-50 Sv2A-53 Sv2A-152 Sv2B-27	Sv2A-95 Sv2B-138		

Sexing by PCR analysis

From the 41 previously unsexed data, 20 individuals were found to be female and 21 were found to be male. An example of a sexing PCR can be seen in Figure 1.

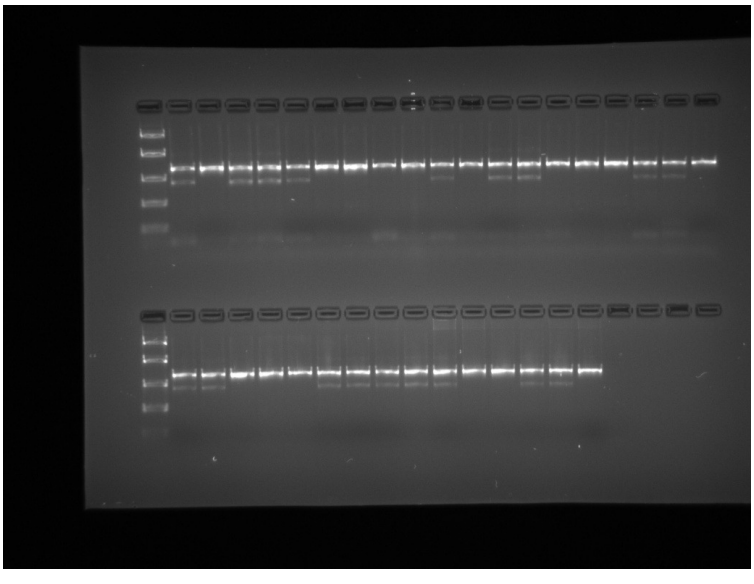

Figure 1. Sex assignment for individuals in the Lord Howe Island Group: two bands denote a female (ZW) and one band denote a male (ZZ). Birds of known sex were used as positive controls in the last four wells: male, female, female, male.

Data Analysis

The mean level of observed and expected heterozygosity, mean number of observed and effective alleles and private alleles are shown in Table 2. The Kermadecs had noticeably less private alleles and a lower number of observed alleles than either the Lord Howe or Norfolk group; the remaining measures of diversity were similar across groups. Microsatellite variability was lowest at loci Sn2B-100, Ss2b-71 and Ss2b-138 with two alleles and highest at locus BOOB-RM4-C03 with a total of 33 alleles (Table 3). There were noticeable differences in the number of alleles found when these microsatellites were used for the focal species, for instance about a third of the loci have a similar number of alleles, about a third of the loci have a higher number of alleles in the Tasman booby and about a third of loci have a lower number of alleles in than in the focal species (Table 3).

Table 2. Standard genetic diversity indices for three Tasman Booby island groups based on 14 microsatellite loci. Measures include mean observed heterozygosity (*Ho*), expected heterozygosity (*He*), observed number of alleles (*Na*), and number of effective alleles (*Ne*). Numbers in brackets represent population size.

	Kermadecs Island Group (16)					Lord Howe Island Group (30)					Norfolk Island Group (27)				
Locus	*Ho*	*He*	*Na*	*Ne*	*Private alleles*	*Ho*	*He*	*Na*	*Ne*	*Private alleles*	*Ho*	*He*	*Na*	*Ne*	*Private alleles*
BOOB-RM2-F07	0.53	0.42	4	1.72	0	0.30	0.35	4	1.54	0	0.62	0.44	3	1.78	0
BOOB-RM3-F11	0.92	0.88	10	8	1	0.92	0.89	13	8.74	2	0.88	0.88	14	8.45	3
BOOB-RM4-C03	0.94	0.88	14	8.39	3	0.97	0.93	23	14.38	11	0.96	0.93	22	14.21	9
BOOB-RM4-D07	0.8	0.57	4	2.33	0	0.40	0.44	6	1.78	1	0.48	0.51	7	2.03	2

BOOB-RM4-E03	0.5	0.51	3	2.04	0	0.65	0.75	6	3.99	2	0.80	0.69	4	3.18	0
BOOB-RM4-F11	0.5	0.54	3	2.16	0	0.82	0.71	5	3.42	2	0.68	0.63	3	2.71	0
BOOB-RM4-G03	0.75	0.77	7	4.3	0	0.86	0.79	7	4.73	0	0.78	0.82	9	5.40	0
Sn2B-83	0.44	0.48	2	1.93	0	0.60	0.51	3	2.02	1	0.54	0.51	3	2.06	1
Sn2B-100	0.38	0.31	2	1.44	0	0.13	0.12	2	1.14	0	0.37	0.30	2	1.43	0
Ss2b-71	0.5	0.49	2	1.97	0	0.57	0.49	2	1.95	0	0.44	0.48	2	1.93	0
Ss2b-138	0.31	0.40	2	1.68	0	0.67	0.49	2	1.95	0	0.11	0.11	2	1.12	0
Sv2A-26	0.06	0.06	2	1.06	0	0.13	0.13	3	1.14	1	0.07	0.07	2	1.08	0
Sv2A-95	0.31	0.44	3	1.78	0	0.50	0.46	3	1.85	0	0.26	0.29	3	1.40	0
Sv2B-138	0.75	0.76	8	4.24	1	0.61	0.86	12	7.23	2	0.86	0.85	13	6.68	3
Averages	0.55	0.54	4.71	3.08	0.36	0.56	0.56	6.5	3.99	1.57	0.56	0.54	6.36	3.82	1.29

Table 3. Comparison of number alleles found in the focal booby species and in the Tasman booby for 14 microsatellite loci. Numbers in brackets represent the number of individuals used in the study.

Locus	Focal Species	Number of alleles found in focal species	Number of alleles found in Tasman booby (73)
BOOB-RM2-F07	Blue-footed	5^1 (30)	4
BOOB-RM3-F11	Blue-footed	35^1 (31)	7
BOOB-RM4-C03	Blue-footed	35^1 (31)	33
BOOB-RM4-D07	Blue-footed	9^1 (31)	5
BOOB-RM4-E03	Blue-footed	4^1 (31)	6
BOOB-RM4-F11	Blue-footed	8^1 (31)	5
BOOB-RM4-G03	Blue-footed	9^1 (30)	9
Sn2B-83	Blue-footed	4^2 (14)	8
Sn2B-100	Blue-footed	0^2 (24)	2

Ss2b-71	Red-footed	13^3 (282)	2
Ss2b-138	Red-footed	7^3 (282)	2
Sv2A-26	Peruvian	7^2 (27)	4
Sv2A-95	Peruvian	0^2 (27)	3
Sv2b-138	Peruvian	10^2 (27)	12

1 Faircloth et al., 2009, 2 Taylor et al., 2010b, 3 Morris-Pocock 2012.

After Benjamini-Yekutieli correction for multiple tests (k= 91, α= 0.00938; Narum 2006) significant deviation from Hardy-Weinberg equilibrium was found for locus Sv2B-138 in the Kermadecs Island Group only ($p < 0.001$). Subsequent analyses using MICROCHECKER indicated null alleles may be present at one locus (Sv2B-138 at LHI) due to a general excess of homozygotes for most allele size classes; this locus was discarded from final analyses in Chapter 3. After implementing the Benjamin-Yekutieli correction (k= 13, α= 0.01572; Narum 2006) four unique pairs of loci displayed linkage disequilibrium in two populations; Lord Howe Island Group: BOOB-RM4-C03 and Ss2b-138 (p=0.00174), and BOOB-RM4-G03 and Ss2b-138 (p=0.00803); Kermadecs Island Group: Sn2B-83 and Ss2b-138 (p=0.00591) and BOOB-RM4-E03 and Sv2A-95 (p=0.00794).

Chromosome location

I assigned chromosomal locations on the chicken, zebra finch and turkey genomes for 14 loci based on sequence homology and was able to allocate chromosome locations with all loci except BOOB-RM4-D07. The chicken genome contained more similar sequences than either the turkey or zebra finch genomes in megablast, blastn and discontiguous blast options. The addition of the blastn and discontiguous BLAST search algorithms provided additional chromosome locations. The majority of loci that yielded a location in the megablast search engine also gave the same location in either or both of the alternate search engines, along with several loci being assigned locations on the blastn and/or discontiguous search algorithms only (Table 4). As such, results are presented for all search algorithms. The one exception where a locus was not on the same chromosome across genomes when employing all search algorithms was the locus BOOB-RM4-G03. This locus was assigned to chromosome 1 in a blastn of the chicken genome, but also assigned to chromosome 1A on the zebra finch genome in a discontiguous search. The turkey genome had a number of chromosomes

orthologous to the chicken chromosomes, but were numbered differently. In this instance, the chicken chromosome 2 is orthologous to two turkey chromosomes (3 and 6), chicken chromosome 3 is orthologous to the turkey chromosome 2, and chromosome 13 in the chicken genome was orthologous to chromosome 15 in the turkey genome.

A visual map of the microsatellite loci can be seen in Figure 2. If loci (including sex linked loci) were present in all genomes, they were also always present on the same chromosome, but not necessarily in the same gene order. For example, while loci BOOB-RM2-F07 and Sv2A-95 are both on chromosome three in the chicken, zebra finch and turkey genomes, in the chicken genome Sv2A-95 is more proximal than the other sequences in position 22,043,154 base pairs (bp) from the top tip of the chromosome and BOOB-RM2-F07 is at 2,915,685 bp from the tip of the chromosome, with two other loci between them. This contrasts with the zebra finch genome, where the two loci are adjacent, with Sv2A-95 distal to BOOB-RM2-F07 at 3,467,663 bp from the tip of the chromosome and BOOB-RM2-F07 at 4,184,960 bp from the tip of the chromosome.

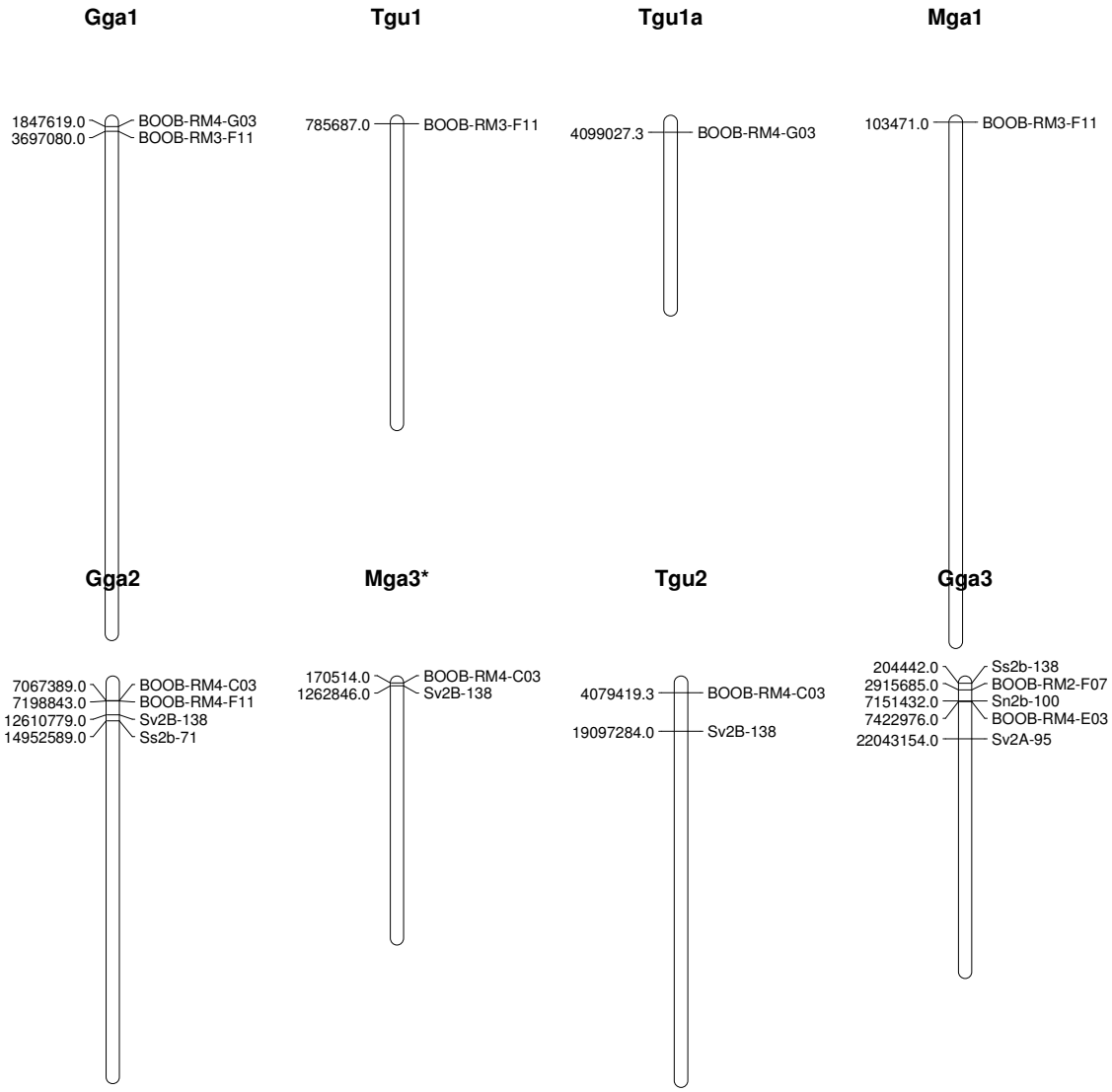

Figure 2. chromosome locations of Blue-footed (*Sula nebouxii*), red-footed *(S. sula*) and Peruvian (*S. variegata)* booby loci in the chicken, turkey and zebra finch genomes. Gga, chicken (*Gallus gallus*), Mga, turkey (*Meleagris gallopavo*), Tgu, zebra finch (*Taeniopygia guttata*) chromosome names. * denote chromosomes in the turkey genome that are orthologous to chicken and zebra finch chromosomes. In this figure, the turkey chromosomes 3, 2, 15 and 17 are orthologous to chromosomes 2, 3, 13 and 15 in the chicken and zebra finch, respectively.

Figure 2 (Continued).

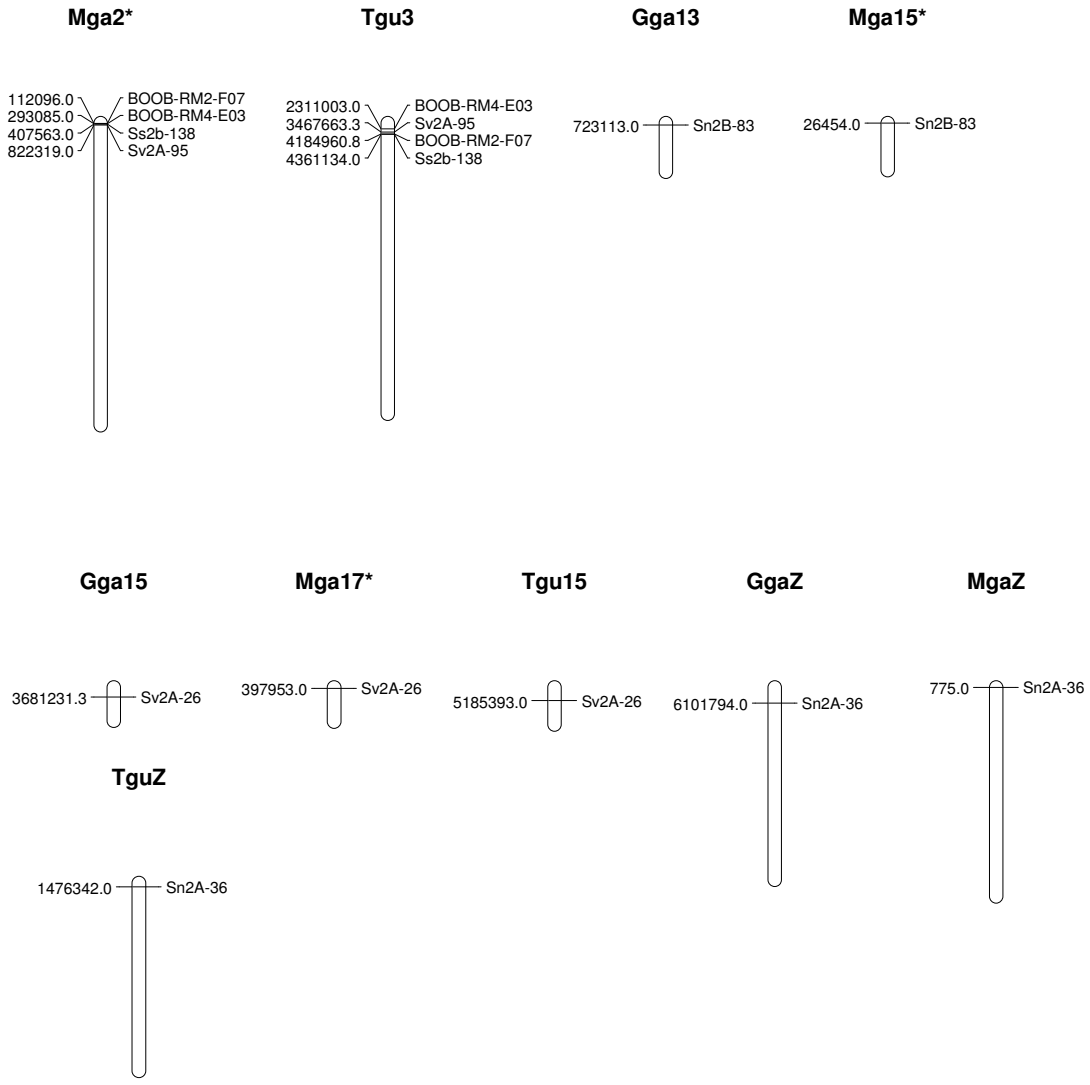

Table 3. Megablast and blastn results of the polymorphic loci against the chicken (*Gallus gallus*), zebra finch (*Taeniopygia guttata*), and turkey (*Meleagris gallopavo*) genomes.

	Chicken						Zebra finch					
	Megablast			Blastn			Megblast			Blastn		
Locus	Chromosome	Length (bp)	E-value	Chromosome	Length (bp)	E-value	Chromosome	Length (bp)	E-value	Chromosome	Length (bp)	E-value
BOOB-RM2-F07	3	741	0	3	821	0	3	350	4.00E-94	3	471	9.00E-131
BOOB-RM3-F11	1	246	6.00E-63	1	363	3.00E-98	NS			1	475	8.00E-132
BOOB-RM4-C03	NS			2	131	3.00E-28	NS			2	373	1.00E-71
BOOB-RM4-D07	NS			NS			NS			NS		
BOOB-RM4-E03	3	588	7.00E-166	NS			3	652	0	NS		
BOOB-RM4-F11	NS			2	271	9.00E-71	NS			NS		
BOOB-RM4-G03	NS			1	93	6.00E-17	NS			NS		
Sn2A-36	Z	569	1.00E-160	Z	580	8.00E-164	Z	580	6.00E-164	Z	580	1.00E-163
Sn2B-83	13	100	8.00E-20	13	127	7.00E-28	NS			NS		
Sn2B-100	NS			3	92	1.00E-16	NS			NS		
Ss2b-71	NS			2	82	8.00E-14	NS			NS		
Ss2b-138	NS			3	131	1.00E-28	NS			3	280	1.00E-73
Sv2A-26	15	239	6.00E-61	15	269	3.00E-70	15	257	2.00E-66	15	309	5.00E-82
Sv2A-95	NS			3	113	4.00E-23	NS			3	358	7.00E-97
Sv2B-138	2	176	4.00E-42	2	221	1.00E-55	2	165	1.00E-38	Doesn't specify	269	4.00E-70

Table 3 (Continued)

Locus	Turkey Megablast			Blastn		
	Chromosome	Length (bp)	E-value	Chromosome	Length (bp)	E-value
BOOB-RM2-F07	2	702	0	2	798	0
BOOB-RM3-F11	NS			1	334	2.00E-89
BOOB-RM4-C03	NS			3	342	3.00E-28
BOOB-RM4-D07	NS			NS		
BOOB-RM4-E03	2	579	4.00E-163	2	639	0
BOOB-RM4-F11	NS			NS		
BOOB-RM4-G03	NS			NS		
Sn2A-36	Z	569	1.00E-160	Z	576	1.00E-162
Sn2B-83	15	76.8	1.00E-12	15	107	6.00E-22
Sn2B-100	NS			NS		
Ss2b-71	NS			NS		
Ss2b-138	NS			2	132	4.00E-29
Sv2A-26	17	241	2.00E-62	17	291	1.00E-76
Sv2A-95	NS			2	129	5.00E-28
Sv2B-138	NS			3	185	1.00E-44

Discussion

I found all loci used that were originally developed for other booby species to amplify, and of those 15 were polymorphic in the Tasman booby *Sula dactylatra tasmani*. One of these loci, however, was found to be sex linked and thus could not be used for downstream genetic analysis of the species.

Nine of these loci were originally developed for the blue-footed (*S. nebouxii*) booby, two for the red footed (*S. sula)* booby, and three for the Peruvian (*S. variegata)* booby. These results provide a good example of successful cross amplification. Masked boobies and red-footed boobies are estimated to have shared a most recent common ancestor approximately 6 million years ago (Mya) (Patterson et al. 2011). In comparison, masked, blue-footed and Peruvian boobies last shared a common ancestor approximately 2 Mya (Patterson et al. 2011).

Thus, this study supports previous claims of more successful cross-amplification with closely related species (Primmer et al. 2005, Salmona et al. 2010). However, two loci developed for the red-footed booby were polymorphic in the Tasman booby. Additionally, Morris- Pocock et al. (2011) used microsatellites originally developed for blue-footed and Peruvian boobies to investigate the phylogeography of brown boobies, grouping this species into four distinctive groups. Dawson et al. (2010) developed 33 polymorphic loci from similar sequences in the chicken and zebra finch, that is, birds from different orders. They found that these loci expressed high utility in passerine birds, along with non-passerines such as shorebirds. Therefore, while researches should aim to use loci developed within a genus from the most closely related species, they should also not disregard loci developed for other species in the same genus if present, especially if studies concern an endangered focal species where time is of the essence.

The discovery of a sex-linked locus that has been used previous research (Taylor et al. 2010a, b, Taylor et al. 2011a, b) highlights the need for constant vigilance when implementing loci already developed; researchers should make an effort to sex their birds when using microsatellite markers. This is an essential precaution to take, as most downstream analyses of microsatellite loci assume such markers are biparentally inherited, (i.e. autosomal) (Ballard and Whitlock 2004). Deviations from this assumption can lead to misrepresentation of the data. It is additionally important to test for truly independent loci by ensuring they are autosomes, do not deviate from Hardy-Weinberg equilibrium and are not physically linked with other loci. Again, if researchers do not check that these assumptions of microsatellites are fulfilled, data analyses may be inaccurate and not true representations of the data, as a higher statistical power can be represented. If two loci are

physically or genetically linked and therefore not independent, they are made redundant as one microsatellite and should not be counted as two. Overlooked linked loci will be analysed assuming independence, which is a false representation of the data; thus, it is important to determine the independence of a microsatellite intended for population genetics research

General diversity indices

From the 14 polymorphic loci found in Table 1, there was more allelic diversity in the Lord Howe and Norfolk Groups when compared to the Kermadecs; however, this may be due to the smaller sample size of 16 individuals in the Kermadecs Group compared to 30 in the Lord Howe Island Group and 27 in the Norfolk Island Group. There was a lack of significant deviation from Hardy-Weinberg equilibrium for one locus in one population, possibly due to small sample size. Additionally, the few pairs of loci found in significant linkage disequilibrium were not consistent across populations (also see below). These combined data suggest that the 14 microsatellite loci cross-amplified in this study are appropriate for additional population genetic analyses in the Tasman booby (see Chapter 3).

Chromosomal locations

In this study I was able to successfully allocate 14 polymorphic loci (including the previously removed sex-linked locus) to chromosomes based on sequence homology in the chicken, zebra finch and turkey genomes. To my knowledge, this is the first study to map microsatellite sequences on three separate avian genomes. All loci bar one (BOOB-RM4-D07), were successfully allocated to a chromosome in at least one genome.

This study provides further support for high synteny in avian genomes. If one locus was found in more than one species, it would always appear on the same chromosome. There was one exception to this with the locus BOOB-RM4-G03, which was located on chromosome 1A in the zebra finch genome, while in contrast it was located on chromosome 1 in the chicken genome. However, on closer inspection, the locus does appear to have remained in the same place, as the zebra finch karyotype has a smaller chromosome 1 than the chicken, and lacks a centromere. The locus is found at the tip of the chromosome in both species, and as such the 1A chromosome in the zebra finch has

been considered homologous to chromosome 1 in the chicken genome. As previous research has highlighted the well conserved synteny between these two species (Warren et al. 2010), and well conserved synteny across avian genomes has been illustrated (Backstrom et al. 2006, Backstrom et al. 2008, Derjusheva et al. 2004, Hale et al. 2008), this study adds to previous research and is reassuring for researchers aiming to find the chromosomal location of microsatellites in the avian genome. This situation is different to the initial differences observed when comparing the turkey BLAST results with the chicken and zebra finch findings. While loci appeared to be on the same chromosomes in the chicken and zebra finch genomes but not in the turkey genome, the loci were actually on the same homologous chromosomes that had been numbered consistently in the chicken and zebra finch genomes but differed in the turkey genome (Dalloul et al. 2010).

In contrast with the conserved synteny of this study, gene order does not appear to be maintained across species. For example, loci BOOB-RM2-F07 and Sv2A-95 were close to twenty million bases apart with two other detected loci between them in the chicken genome, yet were less than a million bases apart and adjacent on the zebra finch genome. This finding coincides with previous studies, where if a researcher is locating a marker across genomes, it can be found at different locations on the same chromosome between species, even if only two genomes are utilised (Backstrom et al. 2010, Hale et al. 2008, Itoh et al. 2006). While previous studies have used sequence mapping to determine location of loci and detect linkage (Dawson et al. 2012), in light of the findings of this study, it is suggested that researchers should use sequence mapping with caution. If the genome of the focal species is not sequenced, BLASTing the markers could help indicate which chromosome the locus is on. However, there would be some hesitation when providing a precise location of the marker. Additionally, researches should endeavour to BLAST sequences on at least two genomes. In this case, it can be stated with confidence which chromosomes on the Tasman booby genome most of the sequences are, but specifically where on the chromosomes remains unknown. Additionally, it is unknown how many chromosomes are present in the Tasman booby karyotype; however, even if there are a strikingly different number of chromosomes across genomes, regions on different chromosomes are likely to be homologous (Dalloul et al. 2010).

The genome mapping gave surprising results when investigating linkage disequilibrium. One locus in particular (Ss2b-138) was found to be in significant linkage disequilibrium with BOOB-RM4-C03, BOOB-RM4-G03 and Sn2B-83 for one population only. Furthermore, according to the mapping results, all these loci are located on different chromosomes; these combined data suggest physical linkage is unlikely. One other pair of loci were located on the same chromosome according to the BLAST results, but this pair were only found to be in linkage disequilibrium in the Kermadecs group.

A previous study (Dawson et al. 2012) suggested that if any loci were mapped <5 Mb apart, one should be discarded to eliminate the possibility of linkage. Conversely, many of the loci in this study were mapped closely to other loci; in fact, all loci were mapped only at the tip of the chromosomes. Yet none of the closely mapped loci appear to be linked. However, the Dawson et al. (2012) paper was comparing sequences in the house sparrow and the zebra finch, which are both passerines and closely related. While this study included genomes from a passerine and two galliformes. Therefore, Dawson's cut off may be applicable for their study, but in more distantly related birds a cut off may not aid the study, as some loci pairs can be closely located in one genome but then be much farther apart in another.

Overall, I found 14 independent polymorphic loci developed for other booby species that are appropriate for future population genetics research in the Tasman booby. The conserved synteny but not conserved gene order is consistent with previous studies. In conclusion, cross-amplification of microsatellite markers is a useful practice in population genetics, however this study also highlights how genomic information can be used to inform marker choice.

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Chapter 3 - Conservation genetics of the rediscovered Tasman Booby, *Sula dactylatra tasmani*

Introduction

The field of conservation genetics is a relatively new one. It is the practice of investigating the genetics of species at risk to aid in their conservation (Frankham 2010). The field encompasses a variety of genetic issues that can be a potential threat to the long-term survival of a species. In particular, one needs to consider the genetics of small isolated populations; when gene flow is restricted such populations become susceptible to genetic drift and inbreeding (Frankham 1998). While genetic drift leads to the random fixation of alleles and decreased heterozygosity (Nei et al. 1975), inbreeding results in the random fixation of alleles and increased homozygosity (Frankham 2003). After this, the fixation of deleterious recessive alleles and increased homozygosity may lead to an expression of deleterious recessive alleles manifested as inbreeding depression (Frankham 2003).

For widely distributed vulnerable species, knowledge of the amount of genetic structure and if there has been a change in effective population size over time can be imperative to species' management and/or recovery. For instance, estimates of genetic structure can be used to determine the appropriate number of management units for a threatened species (Palsbøll et al. 2007), while estimates of short and/or long-term changes in the effective population size can be used to guide effective conservation management strategies for a species at risk (Luikart et al. 2010).

Genetic structure is a measure of the extent of genetic differentiation among populations (Rousset 2004). Genetic structure may be estimated for the entire range of a species (i.e., on a global scale) or among neighbouring populations of a species (i.e. on a local scale). For example, Morris-Pocock et al. (2010b) examined both local and global population genetic structure in brown and red-footed boobies by surveying mitochondrial control region sequence variation in samples collected throughout each species' pantropical range. From this the authors found that while both species exhibit strong global population genetic structure, only brown boobies display strong population genetic structure on a local scale. A subsequent study based on nuclear intron sequence data and microsatellite genotype data corroborated these findings in brown boobies (Morris-Pocock et al. 2011).

The presence or absence of genetic structure in a threatened species may have different conservation implications. For example, if a widely distributed species does not exhibit genetic structure, it may be recommended that the species be treated as a single panmictic population and conservation efforts should be maintained simultaneously across populations (Palsbøll et al. 2007). This is seen in the red throat emperor fish (*Lethrinus miniatus)*, where analysis of eight microsatellite markers from six locations in three geographic regions of the Great Barrier Reef showed no evidence of population genetic structure from all locations sampled (Van Herwerden et al. 2003). This was indicative of high levels of gene flow, and it has been recommended that the Great Barrier Reef be treated as one panmictic population (Van Herwerden et al. 2003). Conversely, if a species exhibits high levels of genetic structure, each population should be treated as a separate management unit and should be monitored at different intervals (i.e., all populations can be monitored separately, as opposed to some populations that need to be monitored at the same time; for example, the Mojave desert tortoise (*Gopherus agassizii),* Hagerty and Tracy 2010). Knowledge of genetic structure is of great importance to conservation biology, as gene flow upholds the genetic diversity needed for the process of evolution (Frankham et al. 2010). If a species exhibits population genetic structure, it suggests that gene flow is restricted among populations.

Genetic structure over large spatial scales can be indicative of a species' dispersal ability: species capable of large scale dispersal may demonstrate less genetic structure. For example, the Canada lynx (*Lynx canadensis*), a mammal capable of high dispersal, has low genetic differentiation among populations ranging across mainland North America (Row et al. 2012). This pattern can also be seen repeatedly in marine studies. For example, Van de Putte et al. (2012) found no global genetic structuring among populations of a highly dispersive mesopelagic fish *Electrona antarctica*, endemic to the Southern Ocean. This suggests high connectivity between populations of this species and gene flow. Additionally, seabird ticks from the Cape Verde Archipelago have been found to have genetic structure among but not within ocean basins due to the trans-oceanic movements of their seabird hosts (Gomez-Diaz et al. 2012).

However, despite high dispersal potential, many species can still exhibit strong levels of genetic structure. For example, two closely related northeastern Pacific gastropods which had identical larval dispersal potential had markedly different patterns of post-glacial structure (Marko 2004). While *Nucella ostrina* showed limited population substructuring in the northern half of its range, low haplotype diversity, and no relationship between genetic differentiation and geographical distance, *N. lamellose* showed significant subdivision between northern (Alaska and northern British Columbia) and southern (Washington, Oregon and southern British Columbia) populations and

exhibited a significant relationship between genetic differentiation and genetic distance among sites. One study on the great frigatebird (*Fregata minor*) and Nazca booby (*Sula granti*), which have similar distributions in the Galapagos, found that while the former species' populations did not show any genetic structure, the Nazca booby populations were substantially genetically differentiated, even within the relatively small geographic scale of the Galapagos archipelago (Levin and Parker 2012). These studies highlight the need to estimate the population genetic structure of a species with high dispersal potential, as such potential does not necessarily lead to a lack of genetic structure.

When studying genetic structure at any level of a species, the use of multiple independent genetic markers is likely to provide the best indication of the extent of genetic differentiation among populations. Different markers can inform researchers about different aspects of a species' conservation, for instance mitochondrial sequence data can inform researchers about historical population size and female mediated gene flow as mitchondrial DNA is predominately maternally inherited (Selkoe and Toonen 2006) while rapidly evolving nuclear markers such as microsatellites are biparentally inherited and can be used to estimate recent patterns of gene flow, detect migrants or determine if a species has gone through a recent bottleneck (Dow and Ashley 1998, Spencer et al. 2000, Maudet et al. 2002 but see Zink 2010). Combined, nuclear and mitochondrial data can tell us more than either marker can alone, making it useful to incorporate both. Burg and Croxall (2001) used both mitochondrial control region sequences and nuclear microsatellites to research population genetic structure of black-browed (*Thalassarche melanophris*) and grey-headed albatrosses (*T. impavida*). While both the mitochondrial and microsatellite data found the grey-headed albatross is globally panmictic, the black-browed albatross was found to have conflicting patterns between the markers. Namely, pairwise estimates of genetic differentiation based on the mitochondrial data were up to 10-fold higher than those based on microsatellite data. This drastically higher estimate of differentiation in a marker that is derived from the female line, combined with a male bias in dispersal, led Burg and Croxall (2001) to attribute such discrepancies to male-mediated gene flow.

Effective population size (N_e) is also an important aspect to consider when investigating the conservation genetics of species at risk. Effective population size is the size of an ideal population which has the same rate of change of heterozygosities or allele frequencies as the observed population (Fisher 1930, Luikart et al. 2010). While difficult to measure, effective population size is worth analysing as it can provide an insight into how large the population is on a genetic scale and ultimately help predict the extinction risk of populations, which is invaluable when trying to

conserve or manage a species. Thorough investigation is needed, however, as miscalculations can be detrimental to conservation efforts. For instance, if a N_e value is grossly overestimated, it could result in a delay to management which in turn could lead to excessive loss of genetic variation and in the worst case scenario, extinction (Welch et al. 2012). It is also important to determine if there has been a reduction in N_e recently. If a species or population already has a low N_e, then it may have gone through a bottleneck and have limited genetic variation or inbreeding depression which can increase a population/species' probability of becoming extinct (Soule and Mills 1998). Additionally, it is important to know whether a small population has always existed at such a low frequency, or whether this population is now small as a result of a population bottleneck, from either the relatively distant past, the relatively recent past, or both.

Masked boobies (*Sula dactylatra*) are large plunge-diving seabirds that breed on oceanic islands in a pantropical distribution and display high natal philopatry (Steeves et al. 2005a, Steeves et al. 2010). The masked booby has four subspecies, one of which, the Tasman booby (*S. d. tasmani*) was recently rediscovered via comparison of modern and ancient mitochondrial control region sequences (Steeves et al. 2010). The Tasman booby is different from other masked booby subspecies in a number of ways; firstly, their sepia coloured irises were discovered by O'Brien and Davies (1990) and later confirmed by Ismar et al. (2010). This eye colour is a feature that separates this subspecies from other subspecies of masked booby which all have yellow irises. *S. d. tasmani* have also been found to have a larger wing span than *S. d. personata* and *S. d. bedouti* (O'Brien and Davies 1990). However, comparisons among the Tasman booby populations are limited, as most studies involve individuals taken from only one island (Ismar et al. 2010); so far little if anything is known about within subspecies variation. Additional morphometric data for the Tasman booby could inform researchers of any differences in the subspecies itself, as morphological differences can be indicative of underlying gene flow restrictions (Dearborn et al. 2003, Steeves et al. 2005b).

While the masked booby is a species of least concern (global population approximately 200,000 birds; IUCN 2012), the Tasman booby is listed as nationally vulnerable in Australia (Garnett and Crowley 2000) and nationally endangered in New Zealand (Miskelly et al. 2008). This subspecies has the smallest number of breeding pairs relative to the other three subspecies, with estimations of fewer than 500 breeding pairs on Lord Howe Island, 350 breeding pairs on Norfolk Island and 100 breeding pairs on the Kermadec Islands (Priddel et al. 2005). Approximately 3,163 nestlings were banded in the Norfolk Island group from 1981-2007 (Priddel et al. 2010). These banding data indicate that there is movement away from the Norfolk island group, particularly of juveniles, but

there is no evidence of natal dispersal (i.e., a bird banded as a nestling on one island group found reproducing on an island group) (Priddel et al. 2010).

Previous studies based on mitochondrial DNA sequence data (Steeves et al. 2003, Steeves et al. 2005a, b) have suggested that there is significant population genetic structure in masked boobies, and there is also some indication of population genetic structure in the Tasman booby (Steeves et al. 2010). However, the sample size for two of the three island groups used was small and sampling was restricted to one molecular marker (i.e., mitochondrial control region sequences). In other words, while there have been studies conducted on masked boobies at a global scale, limited research has been conducted at a local scale. Additionally, morphometric differences can be indicative of underlying restrictions in gene flow. For example, Dearborn et al. (2003) found that three morphometrically distinct central Pacific populations of the great frigatebirds (*Fregata minor*) exhibited significant genetic differentiation despite extensive interisland movements. Therefore, a more extensive study determining the structure and any morphological differences in this subspecies could aid the conservation management of this species.

In this chapter, I use mitochondrial control region sequence data and nuclear microsatellite genotype data to estimate the level of population genetic structure among the three island groups of Lord Howe, Norfolk and the Kermadecs and to determine whether there has been a change in effective population size of the Tasman booby over time. I combine these genetic data with morphometric data to determine the appropriate number of management units to assist in the conservation management of this species. For example, if the Tasman booby populations are not genetically different then they should be categorised as a single management unit and coordinated conservation management efforts between Australia and New Zealand may be appropriate. Additionally, if there is evidence of a drastic reduction in effective population size (i.e., a genetic bottleneck), immediate conservation action may be warranted.

Materials and Methods

Sample collection and DNA extraction

Samples were collected by several researchers from each island group between 2007-2010 (see Appendix 1). As detailed in Chapter 2, DNA was extracted from 73 blood samples by either a Chelex protocol (Hillis 1996) or an Invitrogen PureLink Genomic DNA Kit.

Mitochondrial DNA

Laboratory protocols

500 base pairs of the mitochondrial control region (Domain I = 283 bp, Domain II = 217 bp) were sequenced for 35 individuals using species-specific primers (SdMCRL100B, 5'-AATTCGTGGAAGCAGTCACA-3' and SdMCRH750, 5'-GGGAACCAAAAGAGGAAAACC-3' Steeves et al. 2005b) following the protocols in Steeves et al. (2005a). The remaining 38 individuals were sequenced previously (Steeves et al. 2005b, Steeves et al. 2010).

Data Analyses

Mitochondrial control region sequences were aligned manually using Geneious v 5.5.4 (Drummond et al. 2012) Variable sites were confirmed visually by examination of chromatograms. Ambiguous sites were resolved according to Steeves et al. 2005a (Appendix II). Relationships among haplotypes were visualised by constructing a statistical parsimony network using TCS v1.21 (Clement et al. 2000).

Global φ_{ST} and pairwise φ_{ST} for the mitochondrial sequence data were calculated and statistical significance was assessed by randomisation with 10,000 permutations in ARLEQUIN v3.5 (Excoffier and Lischer 2010). Unlike estimates of F_{ST} based on microsatellite data (see below for details), estimates of φ_{ST} based on sequence data are independent of the mutation rate (Kronholm et al. 2010), so φ_{ST} estimates have not been corrected.

To estimate effective female population size (N_f) through time, an extended Bayesian skyline plot was computed using the program BEAST v1.7.4 (http://evolve.zoo.ox.ac.uk/Beast/), which uses a Markov Chain Monte Carlo (MCMC) approach to estimate a Bayesian posterior distribution of effective population size through time from a sample of gene sequences, given a specified nucleotide-substitution model (Drummond et al. 2005). First, jModelTest (Darriba et al. 2012) was run on the mitochondrial control region sequence data to determine the most appropriate substitution model. Second, BEAST default parameters were used excluding the following: the HKY plus invariant results substitution model with empirical base frequencies, following a strict clock using a generic rate for avian mitochondrial control region of 0.075 substitutions per site per million years (see Steeves et al. 2005b) was used, and relevant priors and operators were modified following the recommendations in Heled (2010) (see Appendix III for details). The analysis consisted of one long chain (10,000,000 generations, trees sampled every 10,000 generations, and 1,000 trees discarded as burn-in). The trend plots and estimated sample size (ESS) of all parameters were monitored for convergence in Tracer v 1.5 (Rambaut and Drummond 2007).

To calculate the N_f from this data, median estimates were multiplied by the number of generations in the last million years (i.e., assuming a generation time of 10 years for masked boobies, median estimates were multiplied by 100,000 generations). The final analysis was repeated three times with different random starting seeds.

Microsatellites

Laboratory protocols

Polymorphic chain reactions (PCRs) were carried out on 13 loci run for all 73 individuals using the protocols outlined in Chapter 2.

Data analyses

Diversity indices for each locus such as observed (*H*o) and expected (*H*E) heterozygosities, mean number of observed alleles and effective number of alleles, and deviations from Hardy-Weinberg equilibrium were calculated in Chapter 2.

Population Structure

Global F_{ST} and pairwise F_{ST} for the microsatellite genotype data were calculated and statistical significance was assessed by randomisation with 10,000 permutations in ARLEQUIN v3.5 (Excoffier and Lischer 2010). Because of the dependency of within-population diversity that F_{ST} can have (Meirmans and Hedrick 2011), a corrected F'_{ST} value was also calculated to correct for this. Additionally, F'_{ST} is well suited for my analyses as it is suited for inferences of the influence of demographic processes such as migration and genetic drift on population structure (Meirmans and Hedrick 2011). A principal component analysis (PCA) was computed in GenAlEx v6.41 (Peakall and Smouse 2006).

To further asses population structure, a Bayesian clustering method was employed via the program STRUCTURE v2.3.4 (Pritchard et al. 2000). STRUCUTRE uses multilocus genotypes to infer the faction of population/individual genetic ancestry (such as the individual sampled, their parents and/or grandparents) that belongs to a cluster (K, Evanno et al. 2005). I performed 10 independent runs (100,000 steps, 10,000 burn-in) with different random seeds for each K value at 1-6. The first set of analyses was run no *a priori* information about the population of origin for each individual, but a second set was run with sample location used as *a priori* information as recommended by Pritchard et al. 2010 for data sets with weak structure (see Results). Results were averaged for each value of K and the value of K that best explains the data was determined by comparing corrected estimates of the posterior probability, denoted L (K), for each value of K. The ad hoc statistic ΔK as described in Evanno et al. (2005) was not calculated because it is not an appropriate test when K =1 (see Results).

MICROCHECKER v2.2.3 (Van Oosterhout et al. 2004) was used to determine if each locus followed the Stepwise Mutation Model (SMM, Ohta and Kimura 1973). To determine if the Tasman booby has undergone a recent genetic bottleneck, I used the program BOTTLENECK v1.2.02 (Cornuet and Luikart 1996, Piry et al. 1999). BOTTLENECK operates by comparing allelic diversity and heterozygosity. Allelic diversity decays more rapidly than heterozygosity after a population has experienced a recent decrease; hence heterozygosity excess can be used to detect recent bottlenecks. Several loci did not

follow the SMM model (see Results), so I used the two-phase model (TPM), which incorporates elements from both the SMM and the Infinite Alleles Model (Cornuet and Luikart 1996).

The software LAMARC (v 2.1.8; Kuhner 2006) was jointly used to estimate theta ($\Theta = N_e\mu$; where N_e is the effective population size and μ is the mutation rate per generation) and the rate of long-term population growth (*g*) in the Tasman booby (Luikart et al. 2010). Each analysis was run in Bayesian mode following the stepwise mutation model, using loci that followed the SMM only (see Results). The upper and lower bounds on the logarithmic prior for theta were 10 and 0.1, respectively, and the upper and lower bounds on the uniform prior for growth (*g*) were 10 and -10, respectively. The analysis consisted of one short chain with 5,000 generations, with trees sampled every 20 generations, with an additional number of 1,000 trees discarded as burn-in, and one long chain with 100,000 generations, with trees sampled every 100 generations and a burn-in of 20,000 trees. The trend plots and estimated sample size (ESS) of all parameters were monitored for convergence in Tracer v1.5 (Rambaut and Drummond 2007). The final analysis was repeated three times with different random starting seeds.

Morphometric data

Unpublished data for Lord Howe Island Group and Norfolk Island Group were provided by P. O'Neill and J. Sommerfeld, respectively; these were combined with published data from the Kermadecs Island Group (Ismar et al. 2010). Measurements of the culmen, tarsus and wing length of both male and female Tasman boobies were provided from Lord Howe Island (22 males and 12 females) and Kermadecs (five males and two females) samples. Measurements of the culmen and wing length for both sexes were provided for Norfolk samples (15 males and 6 females). Masked boobies, including the Tasman booby, exhibit reverse sexual dimorphism (Weimerskirch et al. 2009), thus, mean and standard deviations for each morphometric measurement were calculated for males and females separately. Sample sizes were too small to warrant comparative statistical analysis.

Results

Mitochondrial data

A total of 14 haplotypes defined by 25 variable sites were found in 73 samples, two of which were unique to this study (Nor_77 and Nor_254) and found in one individual only (Table 1). The most frequent haplotypes were Sd_35 and Sd_36, both of which were found in all three island groups (Table 1). From the statistical parsimony network (Figure 1), there are two main clades separated by six steps. Both of these clades contain haplotypes from all three island groups; however one clade contains relatively more haplotypes from the Norfolk and Kermadecs Island Groups whereas the other contains relatively more haplotypes from the Lord Howe Island Group. Haplotype Sd_37 could not be connected to the network as it was beyond the 95% connection limit.

Table 1. Variable sites among Tasman booby cytochrome *b* haplotypes. LHI - Lord Howe Island Group, NOR - Norfolk Island Group, KER - Kermadecs Island Group

Haplotype	Population Group			Variable sites																								
	LHI	**NOR**	**KER**	**4**	**16**	**17**	**18**	**27**	**29**	**30**	**57**	**60**	**67**	**97**	**112**	**117**	**120**	**140**	**148**	**156**	**161**	**185**	**198**	**199**	**213**	**231**	**250**	**435**
Sd_35	4	15	3	G	C	C	T	C	C	T	C	C	G	G	T	T	T	T	C	G	G	C	C	T	G	G	C	G
Sd_36	17	3	2	.	T	T	C	.	.	C	.	.	A	.	.	C	.	.	.	A	.	.	.	C	.	A	.	.
Sd_37	1			A	.	T	C	T	.	.	.	.	.	A	C	C	C	.	T	.	.	.	T	.	A	A	.	.
Sd_38	1			.	T	T	C	.	.	.	.	.	A	.	.	C	.	.	.	A	.	.	.	C	.	A	.	.
Sd_39	4			.	T	T	C	.	.	C	.	T	A	.	.	C	.	.	.	A	.	.	.	C	.	A	.	.
Sd_40	1			.	T	T	C	.	.	C	.	.	A	A	.	C	.	.	.	A	.	.	.	C	.	A	.	.
Sd_41	1	4	7	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Sd_42	1		1	.	T	T	C	.	.	C	.	.	A	.	.	C	.	.	T	A	.	.	.	C	.	A	.	.
Ker_11			2	.	T	T	C	.	.	C	.	.	A	.	.	C	.	.	.	A	A	.	.	C	.	A	.	.
Ker_13			1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	T	.
Nor_3		2		.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Nor_7		1		.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
Nor_77		1		.	T	.	.	.	.	.		.		A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Nor_254		1		.	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	A	.	T	.	.	.	A	.	.
Total	30	27	14																									

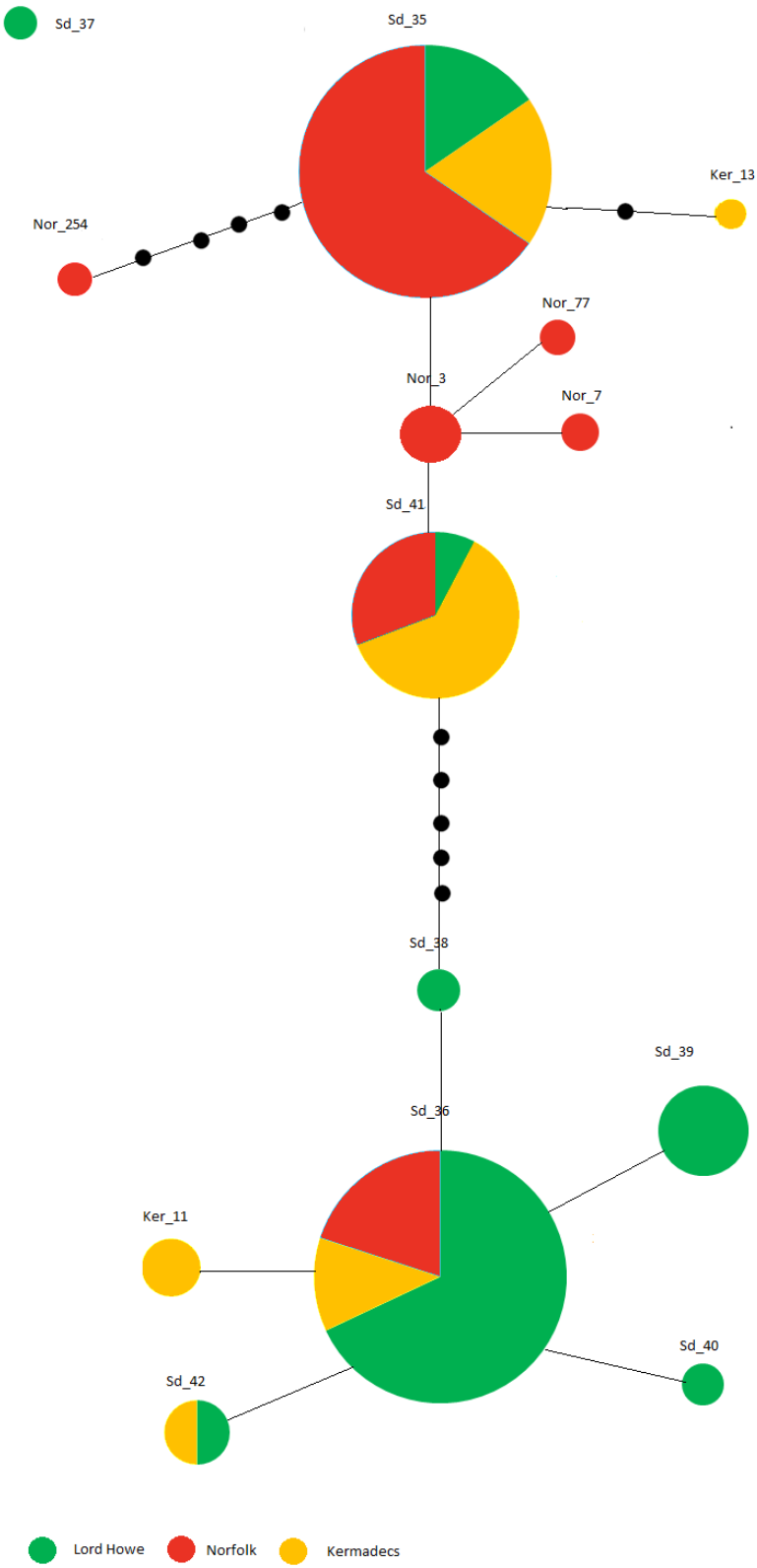

Figure 1. Statistical parsimony network of the control region haplotypes in population groups of the Tasman booby. Circle sizes are proportional to the frequencies of the haplotype. Filled circles represent missing haplotypes.

The extent of genetic differentiation among the three island groups for the mitchondrial DNA data was high and significant (φ_{ST} = 0.40, p <0.000). Pairwise φ_{ST} values are compiled in Table 2, with significant genetic differentiation between all island groups, with the most differentiation between Lord Howe Island and the Norfolk Island groups (φ_{ST} = 0.54, p <0.000).

Table 2. Pairwise estimates of genetic differentiation for microsatellite genotype data (a) and mitochondrial sequence data (b) in the Tasman booby. (a) Pairwise F_{ST} estimates below the diagonal, and pairwise F'_{ST} estimates above the diagonal. (b) Pairwise Φ_{ST} estimates below the diagonal. It is unnecessary to correct estimates of Φ_{ST}, see text. Estimates in bold are significant at P < 0.05.

(a)

	Kermadecs	Lord Howe	Norfolk
Kermadecs		**0.048**	**0.081**
Lord Howe	**0.022**		**0.071**
Norfolk	**0.033**	**0.038**	

(b)

	Kermadecs	Lord Howe	Norfolk
Kermadecs			
Lord Howe	**0.29**		
Norfolk	**0.12**	**0.54**	

The Bayesian skyline plot indicates a stable population size throughout the species' history, with a slight increase in population size approximately 500 years ago (Figure 2). All parameters converged well in each run (i.e., ESS values were above 200 and trend plots looked like "hairy caterpillars" with chains rapidly fluctuating around an equilibrium).

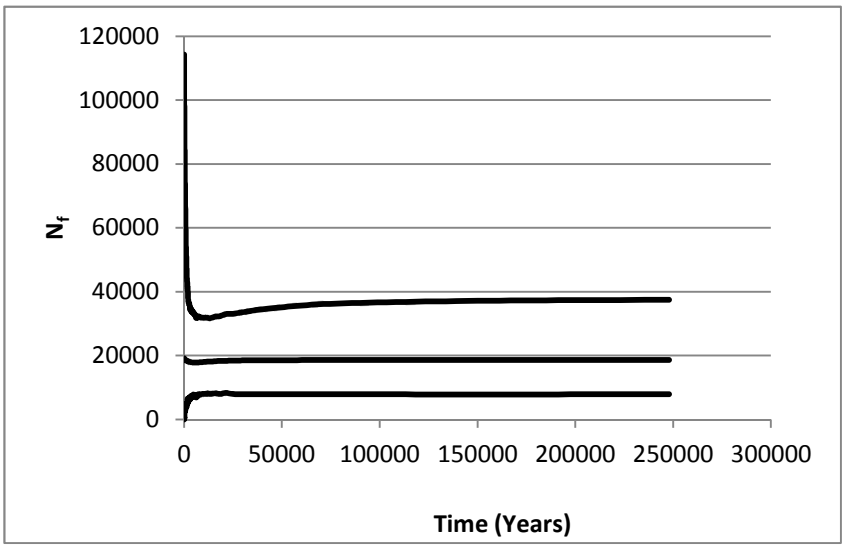

Figure 2. Bayesian skyline plot derived from the control region of the Tasman booby *Sula dactylatra tasmani*. The x axis is in years before present, and the y axis is the effective female population size (N_f). The upper and lower lines represent the upper and lower 95% HPD limits, and the middle line is the median.

Microsatellite data

The extent of genetic differentiation among the three island groups for the microsatellite data was weak but significant, before (F_{ST} = 0.028, p<0.001) and after correction (F'_{ST} = 0.067, p <0.001). Pairwise φ_{ST} estimates can be seen in Table 2, with weak but significant differences between all island groups, with the greatest differentiation between Norfolk Island and the Kermadecs groups (F'_{ST} = 0.08, P<0.000). The PCA indicates weak spatial structuring between the three island groups (Figure 3).

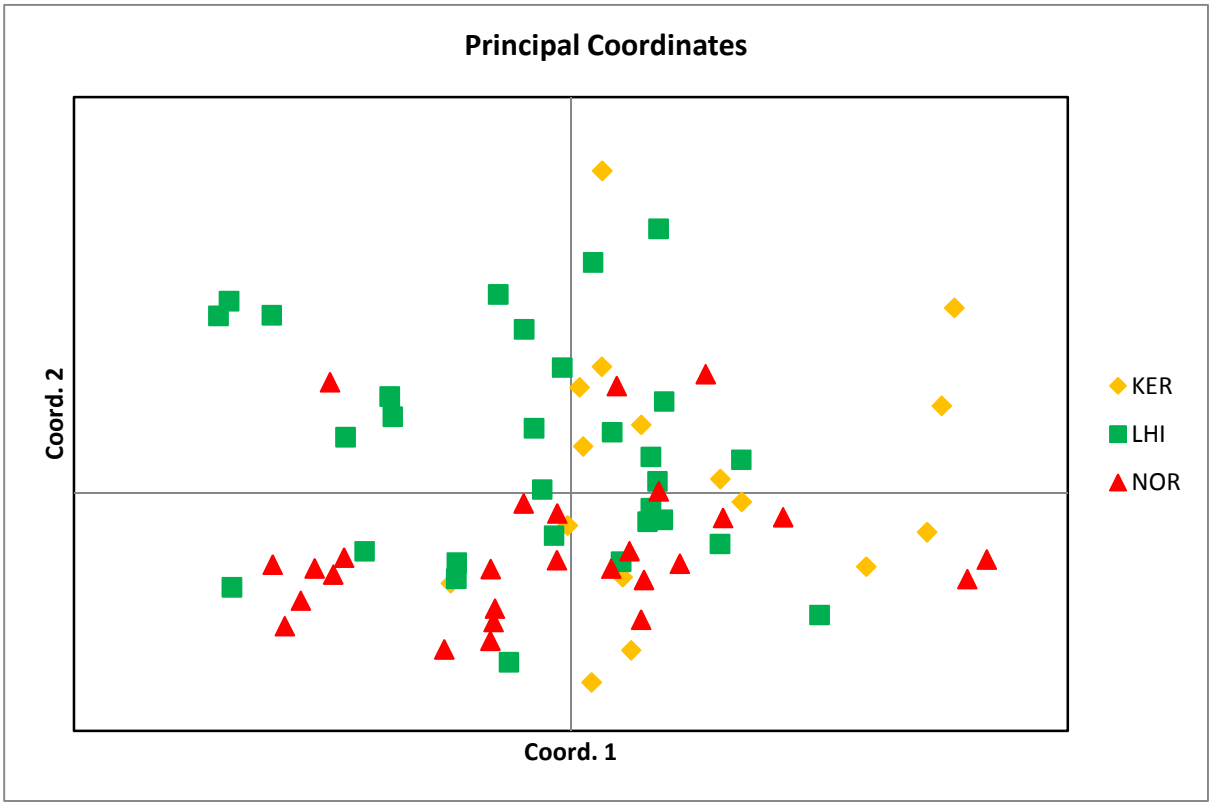

Figure 3. PCA analysis of the three island groups: Lord Howe (LHI), Norfolk (NOR) and the Kermadecs (KER) of the Tasman booby (*Sula dactylatra tasmani*).

When determining the number of clusters using STRUCTURE, both *a priori* and no *a priori* sample location information was used, but as per the recommendation by (Pritchard et al. 2010) for data

sets with weak structure (see above), I present the results for the runs that included *a priori* information only; however, both data sets yielded similar results. Each of the 10 independent replicates for all six values of K yielded consistent results, and the corrected estimates of the posterior probability for K = 1 was higher than any other estimate of K (Figure 4).

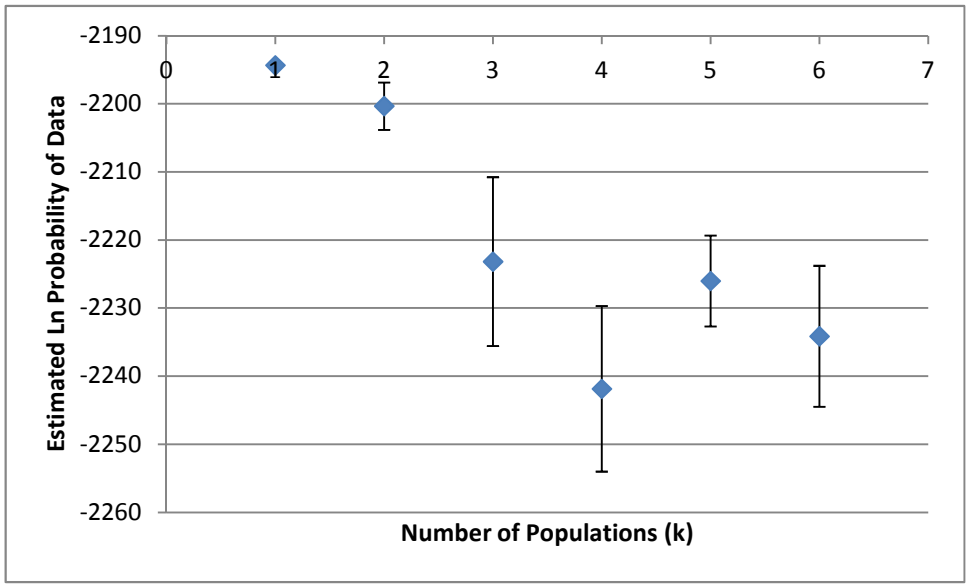

Figure 4. Corrected likelihood values (L) for the number of populations present (K) in the Tasman booby, *Sula dactylatra tasmani*.

To test for evidence of male-mediated gene flow (see below), I repeated the test above but also selected "test for migrants" to ascertain if any of the male individuals from one island group could be assigned to either of the remaining two island groups. However, no individual could be assigned as a first, second or third generation migrant with a posterior probability above 0.95.

Seven of the microsatellite markers were found to be in SMM (BOOB-RM4-E03, BOOB-RM4-G03, Sn2B-83, Sn2B-100, Ss2b-71, Ss2b-138 and Sv2A-95). BOTTLENECK did not detect a signal of a recent population bottleneck in the species as none of the loci showed a relative heterozygosity excess in either the stepwise mutation model or two phase model (SMM P=0.228; TPM P=0.245).

The long term population growth rate estimated by LAMARC, *g*, was -0.15 with upper and lower 95% confidence intervals of -0.28 and -0.09, respectively. Theta was estimated at 1.47 with upper and lower 95% confidence intervals of 4.36 and 0.21, respectively. However, whereas the *g* parameter converged well in each run, convergence was poor in each run for the theta parameter. Thus, whereas the estimate of *g* is very likely to be accurate, the estimate of theta is not. Based on the

equation $\Theta_t = \Theta_{now}e^{-gt}$, where Θ_t is theta at time t, Θ_{now} is the current theta, and *t* is measured backwards in time and is given in mutation units (Kuhner 2006), and assuming a microsatellite mutation rate of 0.0001 (i.e., 1 mutation every 10,000 years), the effective population size of the Tasman booby would have been approximately 16% higher than present 10,000 years ago. Baring in mind that it is unlikely to be correct, based on the equation $\Theta = N_e\mu$, and assuming a mutation rate of 0.0001, the estimate for the current effective population size of the Tasman booby is 14,700 individuals.

Morphometric data

There was a size overlap in wing length among all three island groups for both males and females, but culmen length was smaller for males from the Kermadecs Island Group compared to males elsewhere and tarsus length was smaller for both males and females from the Kermadecs island group compared to Lord Howe Island Group (Table 3). However, it is unclear whether these differences are statistically or biologically significant (that is, because the data was collected by a different researcher at each location, these morphometric differences may be due to measurement error).

Table 3. Mean morphometric measurements of adult Tasman boobies from three island groups.

Island Group, sex	Wing, mm (SD)	Tarsus mm (SD)	Culmen mm (SD)
Kermadecs			
Male (n=5)	459.7	64.13	108.46
	(11.9)	(2.29)	(2.95)
Min, max	[459, 470]	[61.5, 67]	[105.48, 112.65]
Female (n=2)	476.25	66.41	113.59
	(13.08)	(0.01)	(4.06)
Min, max	[467, 485.5]	[66.4, 66.42]	[110.72, 116.46]
Lord Howe			
Male (n=22)	458.95	71.34	113.19
	(8.46)	(2.18)	(3.25)
Min, max	[446, 473]	[66.3, 75.5]	[108.9, 121.2]
Female (n=12)	462.92	73.05	113.2
	(10.17)	(1.67)	(2.65)
Min, max	[443, 484]	[70.7, 75.5]	[109.4, 118.2]
Norfolk			
Male (n=15)	459.6	N/A	111.58
	(6.52)		(1.79)
Min, max	[443, 474]		[109.38, 115.02]
Female (n=6)	469	N/A	111.69
	(9.53)		(1.65)
Min, max	[460, 487]		[108.64, 113.1]

Discussion

Genetic structure

This is the first study to examine the local population genetic structure of the Tasman booby using both uniparentally and biparentally inherited markers. There are differences in the mitochondrial and microsatellite datasets. The microsatellite data suggests that genetic structure is weak. In contrast, the mitochondrial data indicates a high level of genetic structure among island groups, especially between the Lord Howe and Norfolk Island Groups. However, the higher level of structure observed in the mitchondrial data could be due to stochasticity (i.e., because mitochondrial is maternally inherited and haploid, it has a N_e that is one-fourth that of biparentally inherited diploid markers such as microsatellites (Ballard and Whitlock 2004; Zink and Barrowclough 2008)). Indeed, the usefulness of mitochondrial data for avian population genetic/phylogeographic studies has been met with some contention. While Zink & Barrowclough (2008) argue that mitochondrial data is sufficient to answer questions concerning population genetic/phylogeographic patterns, and that "the case for the primacy of nuclear variation for studies of phylogeography is not so clear to us", Edwards and Bensch (2009) respond that to use only one marker for avian population genetic/phylogeographic analyses is illogical as "statistical common sense suggests that when the desire is to make statements about populations or taxa—entities at a higher level of organization than the gene—one must sample multiple loci". In response to this, Barrowclough and Zink (2009) agree that the use of nuclear loci can provide essential contributions to population genetic research; however, they believe that such loci are not as efficient at recognising recent qualitative geographical patterns and most of the time will require additional mitochondrial data. Ultimately, it appears that mitochondrial data is a useful tool for conservation genetics; however, I would not solely rely on one molecular marker for all my analyses when there are additional options that can only add to my findings.

Alternatively, the different levels of genetic structure found in the mitochondrial and microsatellite datasets could be the result of male-mediated gene flow. While females are "known" to be the more dispersive of the sexes in birds (Clarke et al. 1997, Dale 2001), male mediated gene flow has also been suggested in other seabird species. For example, Burg and Croxall (2001) found a similar discrepancy between mitochondrial and microsatellite data in black-browed albatross and attributed this to male-mediated gene flow. However, because these discrepancies could be due to other

factors such as size homoplasy (Karl et al. 2012), future work is needed to determine if male-mediated gene flow is indeed present in the Tasman booby. Introns can be more informative than either mitchondrial or microsatellite data (see Congdon et al. 2000), and additionally, one can compare introns with different inheritance patterns (that is, autosomal and sex-linked introns). In birds, females are the heterogametic sex with a karyotype of ZW, while males are ZZ (Daniel et al. 2007). Comparing introns on the Z chromosome with introns on the autosomes can inform researchers about male-mediated dispersal (Li and Merila 2010).

Overall, it is not suitable to recommend which marker is providing the most "accurate" data in terms of genetic structure, rather future work is required to determine if the discrepancy is biologically meaningful (Karl et al. 2012).

Additionally, the differences between the results for the F-statistics, cluster and principal component analyses conducted on the microsatellite data warrant discussion. Whereas the population cluster analysis suggests the Tasman booby is functioning as one population, the global and pairwise F'_{ST} estimates indicate weak but significant population genetic structure and the PCA analysis also shows weak spatial structuring. However, there is evidence that the STRUCTURE does not perform well when presented with data containing weak population genetic structure (Evanno et al. 2005) and the number of clusters detected by STRUCTURE can be can be strongly influenced by variations in sample size (Kalinowski 2011).

Two aspects of the Tasman booby's morphology that separate it from the other subspecies of masked booby are its longer wings and culmen (O'Brien and Davies 1990). The morphometric data presented in this study suggests there is no apparent difference in wing length among the three island groups, but future work is required to determine if the apparent differences in tarsus and culmen length are biologically meaningful.

Population Size

Previously, the past population size of the Tasman booby was unknown. From this study, the combined analyses data indicate that the subspecies has not gone through a recent genetic bottleneck and existed as a naturally small population at least for the last 25,000 years: whereas the BOTTLENECK analysis suggests that there has not been a reduction in population size in the last few generations, the LAMARC analysis suggests that the population size approximately 10,000 years ago was marginally higher than today and the Bayesian skyline plot suggests that the population size has

been relatively constant for the last 25,000 years. The slight increase in population size indicated by the Bayesian skyline plot approximately 500 years ago is likely an artefact of the analysis but warrants further investigation. Although Bayesian skyline plots based on a single locus should be interpreted with caution (Heled and Drummond 2008, Heled and Drummond 2010), these combined data suggest that the current population size of the Tasman booby reflect its past size.

The current population size estimate for the Tasman booby is approximately 1,900 adults (Priddel et al. 2005). In contrast, estimates of current effective population sizes calculated for both the LAMARC and BEAST analyses are exceedingly high and grossly outnumber the observed number of birds in this subspecies. However, as discussed above, the theta parameter did not converge during the LAMARC analysis and the subsequent estimate of N_e should be interpreted with extreme caution, if at all. Subsequent analyses which include additional microsatellites that follow the stepwise mutation model are warranted. Although all parameters converged during the BEAST analysis, Heled and Drummond (2008, 2010) strongly advise against literal interpretations of effective population size based on a single locus. Further analyses which include additional loci (that is, nuclear intron sequence variation) are well warranted.

Conservation Implications

The combination of both mitochondrial sequence and nuclear genotype data has proved most useful in this study. Whereas there is a lack of congruency between the two markers in regards to the level of population genetic structure, both markers indicate that the population size of the Tasman booby is naturally small and has remained at a relatively stable size for the past 25,000 years. The latter finding suggests that immediate conservation action to increase the population size of the Tasman booby is not warranted, but these data should serve as a useful baseline for future population monitoring.

Despite the incongruency between the two types of markers, the combined data indicate that there is significant genetic structure among the three island groups of the Tasman booby, but none require designation as a separate management unit. Rather, although further research is required to determine if there is indeed male-mediated gene flow in this subspecies, in the interim, the Tasman booby should be managed as a single management unit.

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Chapter 4 – Synthesis

Genetics has been employed to inform the conservation of numerous species of seabirds (Eda et al. 2012, Gangloff et al. 2012, Solovyeva and Pearce 2011, Szczys et al. 2012, Welch et al. 2012). While multiple studies have been conducted on the population genetics of the masked booby (*Sula dactylatra*, Steeves et al. 2005a, b) and other pantropical booby species (Friesen et al. 2007, Morris-Pocock et al. 2010b, Morris-Pocock et al. 2012), little population genetic research has been conducted concerning the Tasman booby subspecies (*S. d. tasmani*) (but see Steeves et al. 2010). As masked boobies are presumed to exhibit high natal philopatry (Steeves et al. 2005b, Steeves et al. 2010), and the Tasman booby is known to differ in appearance from other subspecies of the masked booby in a number of ways (Ismar et al. 2010, O'Brien and Davies 1990), testing for population genetic differentiation on a local scale seemed an obvious area to investigate. In this thesis, I applied population genetic methods to mitochondrial DNA sequence and microsatellite genotype data, and combined it with morphometric data, to test the conservation status of this recently rediscovered threatened seabird.

The Utility of Microsatellite and Mitochondrial Markers

Firstly, this study has uncovered some new insights into the utility of microsatellites that were not developed for the focal species in question. In Chapter 2, I selected and screened 43 microsatellite loci that were developed for three other booby species, all of which amplified in the Tasman booby. From these, I compiled a subset of 15 loci that were found to be polymorphic. Thirteen of these were used for further downstream genetic analyses (two loci were discarded either because it was sex linked, or there was insufficient data). This is an encouraging find, as researchers may not need to expend a large amount of resources to obtain markers suitable for their study species.

Of the 15 polymorphic loci originally compiled, I was also able to locate 14 of them across three avian genomes: the chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*) and the zebra finch (*Taeniopygia guttata*). From this, I found strong evidence to support high synteny across avian genomes: if a microsatellite locus was found in more than one genome, it was always on the same chromosome. This finding supports previous work (e.g. Backstrom et al. 2008, Hale 2008) and suggests that these markers are likely to be present on the same chromosomes in the Tasman booby. Gene order, however, does not appear to be as conserved across genomes. If multiple loci

were detected on the same chromosome in more than one genome, they could be located several million base pairs apart on one genome while they were nearly adjacent on another. This suggests that researchers should not assume an exact location of a locus when using another completely sequenced bird genome as a proxy; in this instance, the precise location of these markers in the Tasman booby genome remains unknown.

The use of mitochondrial sequences for population genetics has recently gone under some debate. Ballard and Whitlock (2004) believe that while mitochondrial DNA has been historically informative in phylogenetic inference, they also state that this marker should be removed from any future phylogenetic studies because of problems with its "natural history", such as effective population size, mutation rates and recombination. The same arguments apply to population genetic and phylogeographic inference. While at the other end of the spectrum, Zink and Barrowclaw (2008) argue that mitochondrial DNA is sufficient enough to describe patterns of avian population genetics and phylogeography on its own, without the added sequence data of other markers. While mitochondrial DNA has its advantages (such as a coalescent time one quarter that of nuclear genes) and its drawbacks (it only represents the female line in most animals), it should neither be discarded nor used as the sole tool for any aspect of conservation genetics, rather it should be used in concert with other independent tools such as nuclear markers. Personally, from this research and others, I believe that no single marker should be used to infer any aspect of the conservation genetics of a species, and researchers should endeavour to use at least one other set of data in their studies, be it molecular, morphological or behavioural. Congruency between data sets increases the confidence that the data sets reflect the same evolutionary history (Rubinoff and Holland 2002). While my data has revealed a magnitude of difference between the markers in both local and global genetic structure, I believe this could be due to the stochasticity in the mitochondrial sequence data. There is also the drawback that while microsatellites have a rapid mutation rate, they can also express homoplasy. Future work could combat this by using nuclear intron sequence data that have similar mutation rates but different patterns of inheritance (e.g., autosomal and sex-linked chromosomes).

Conservation Implications for the Tasman Booby

In Chapter 3, I found weak but significant gene flow across the three island population groups, but no one island group particularly emerged as drastically different in both the mitochondrial and microsatellite data. Additionally, there is a stable population size that had not encountered any

drastic fluctuations in the last 25,000 years. However, there appear to be relatively few individuals of this subspecies compared to the masked booby species. From this, conservation managers now have the knowledge that the Tasman booby exists as a naturally small population, and have a baseline in size as a comparison in case this subspecies ever plummets in size.

However, it is still unknown whether there is male-mediated gene flow in this species. Use of multiple markers to determine male-mediated gene flow has been successful in other marine species (Burg and Croxall 2001, Daly-Engel et al. 2012). As we do not know if either sex exhibits strong natal philopatry, investigation of male-mediated gene flow is what future research should be focused on. This could be achieved with the addition of both autosomal and sex-linked nuclear intron markers. Also, additional, congruent morphological data with sufficient analyses could highlight any differences in size among the underlying groups, which may be indicative of underlying restrictions in gene flow.

It is reasonable to suggest that at this point in time, the Tasman booby should be managed as single management unit, and combined management efforts from Australian and New Zealand authorities are worth considering.

General Summary and Future Directions

This research provides strong support for the conserved synteny of molecular sequences across avian genomes, and provides new insight into the less researched gene order of sequences among birds. In addition, my research revealed population genetic structure in the Tasman booby, but it is unclear whether gene flow is male-mediated. My research also showed that the long-term population size of the Tasman has been relatively stable.

I can conclude that, from a genetics perspective, the Tasman booby is not under immediate conservation threat and occurs at a naturally small population size, unlike other subspecies of the masked booby such as the blue-faced booby *S. d. personata* which is estimated to have half of their entire population on Clipperton Island in numbers exceeding 100,000 individuals (Pitman et al. 2005). However, additional data, in particular sex-lined and autosomal intron sequence data will shed light on whether gene-flow in the Tasman booby is male-mediated and will also provide a much more realistic estimate of the current effective population size of this subspecies.

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Appendices

Appendix I

Samples used

There were 86 individuals sampled for this thesis at different life stages. Several samples were not used in the final analyses for a number of reasons stated below.

Sample Name	Date Collected	Island Group	Life Stage	Sample Discarded?	Reason Discarded
LHI 1	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 2	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 3	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 4	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 5	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 6	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 7	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 8	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 9	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 10	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 11	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 12	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 13	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 14	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 15	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 16	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 17	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 18	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 19	2001	Lord Howe	Chick or Breeding Adult	No	

LHI 20	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 21	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 22	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 23	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 24	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 25	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 26	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 27	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 28	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 29	2001	Lord Howe	Chick or Breeding Adult	No	
LHI 30	2001	Lord Howe	Chick or Breeding Adult	No	
MB 1	2007	Norfolk	Juvenile	No	
MB 2	2007	Norfolk	Chick	No	
MB 3	2007	Norfolk	Juvenile	Yes	Uncertain of hatching site
MB 4	2007	Norfolk	Chick	No	
MB 5	2007	Norfolk	Chick	No	
B 1	2007	Norfolk	Juvenile	Yes	Uncertain of hatching site
B 2	2007	Norfolk	Juvenile	Yes	Uncertain of hatching site
B 3	2007	Norfolk	Juvenile	Yes	Uncertain of hatching site
B 4	2007	Norfolk	Juvenile	Yes	Uncertain of hatching site
B 5	2007	Norfolk	Juvenile	Yes	Uncertain of hatching site
B 6	2007	Norfolk	Juvenile	Yes	Uncertain of hatching site
7	2009/2010	Norfolk	Adult	No	
11	2009/2010	Norfolk	Adult	No	
61	2009/2010	Norfolk	Adult	No	

62	2009/2010	Norfolk	Adult	No	
64	2009/2010	Norfolk	Adult	No	
69	2009/2010	Norfolk	Adult	No	
72	2009/2010	Norfolk	Adult	No	
109	2009/2010	Norfolk	Adult	No	
124	2009/2010	Norfolk	Adult	No	
132	2009/2010	Norfolk	Adult	No	
134	2009/2010	Norfolk	Adult	No	
144	2009/2010	Norfolk	Adult	No	
160	2009/2010	Norfolk	Adult	No	
163	2009/2010	Norfolk	Adult	No	
254	2009/2010	Norfolk	Adult	No	
272	2009/2010	Norfolk	Adult	No	
273	2009/2010	Norfolk	Adult	No	
275	2009/2010	Norfolk	Adult	Yes	Duplicate sample, used as blind control
284	2009/2010	Norfolk	Adult	No	
287	2009/2010	Norfolk	Adult	No	
290	2009/2010	Norfolk	Adult	No	
366	2009/2010	Norfolk	Adult	No	
370	2009/2010	Norfolk	Adult	No	
380	2009/2010	Norfolk	Adult	No	
KER 27	2008	Kermadecs	Juvenile	No	
KER 28	2008	Kermadecs	Juvenile	No	
KER 31	2008	Kermadecs	Juvenile	No	
KER 32	2008	Kermadecs	Chick	Yes	Full family sampled

KER 33	2008	Kermadecs	Juvenile	No	
KER 37	2008	Kermadecs	Adult	No	
KER 47	2008	Kermadecs	Adult	No	
BK 1	2007	Kermadecs	Juvenile	No	
BK 2	2007	Kermadecs	Adult	No	
BK 3	2007	Kermadecs	Chick	No	
BK 4	2007	Kermadecs	Juvenile	No	
BK 5	2007	Kermadecs	Adult	No	
BK 6	2007	Kermadecs	Juvenile	No	
BK 7	2007	Kermadecs	Juvenile	No	
BK 8	2007	Kermadecs	Adult	Yes	Uncertain of hatching site
BK 9	2007	Kermadecs	Chick	No	
BK 10	2007	Kermadecs	Adult	Yes	Uncertain of hatching site
BK 11	2007	Kermadecs	Juvenile	No	
BK 12	2007	Kermadecs	Adult	Yes	Uncertain of hatching site
BK 13	2007	Kermadecs	Juvenile	No	
BK 14	2007	Kermadecs	Adult	Yes	Uncertain of hatching site

Appendix II

Ambiguity in haplotypes

There were six ambiguous sites likely due to heteroplasmy (Steeves et al. 2005a). The ambiguity was resolved as per Steeves et al. (2005a) as follows: if the haplotype sequence with the ambiguous site (e.g. C/T for sample 124 from the Norfolk Island Group at site 168) matched an already existing haplotype as opposed to being a novel haplotype then it was assigned to the already existing haplotype. If a haplotype had an ambiguous site where either base yielded an already existing haplotype (e.g. A/G for sample 61 from the Norfolk Island Group at site 161), the base from the more ancestral haplotype was selected.

Sample	Variable site	Ambiguity	Called as	Second option
124	168	C/T	Sd41	New haplotype
284	83	C/T	Sd41	New haplotype
61	161	A/G	Nor_3	Nor_7
LHI14	94	C/T	Sd40	New haplotype
LHI2	147	A/G	Sd39	New haplotype
LHI26	147	A/G	Sd42	New haplotype

Appendix III

The following priors and operators parameters were altered according to the Extended Bayesian Skyline Plot Tutorial" (Heled, 2010).

Parameter	Default Setting	Altered to
Priors		
kappa	LogNormal [1, 1.25], initial=2	Uniform [0, 10], initial= 2
Operators		
kappa	Weight at 0.1	Weight at 2.0
demographic.indicators	Weight at 30.0	Weight at 35.0
demographic.scaleActive	Weight at 6.0	Weight at 20.0