Evolutionary and conservation genetics of the Seychelles warbler (*Acrocephalus sechellensis*)

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This thesis is dedicated to Laura, my wife and partner-in-crime,

for the endless supply of love, support, tea and cake.

Abstract

In this thesis, I investigated how evolutionary forces and conservation action interact to shape neutral and adaptive genetic variation within and among populations. To accomplish this, I studied an island species, the Seychelles warbler (*Acrocephalus sechellensis*), with microsatellite markers and major histocompatibility complex (MHC) genes as measures of neutral and adaptive variation respectively. First, I used museum DNA and historical records to reveal a recent bottleneck that provides context for the contemporary genetic variation observed in this species. I then determined the impact of four translocations on genetic diversity over two decades. I found that diversity does not differ significantly between islands but the use of smaller founder sizes in two translocations has caused population divergence. These results indicate that stochastic genetic capture is important in translocations and that future assisted gene flow between populations may be necessary. As a tool for conservation practitioners, I wrote a technical report of the most recent translocation - to Frégate Island - detailing practicalities and outcomes to help inform future translocation policy. Using two translocation events as experiments, I then tested whether MHC-based social mate choice acts to maintain MHC diversity in the Seychelles warbler, finding that male age and heterozygosity, but not MHC, predicted pairing success. Lastly, I investigated survival and reproductive consequences of *Ase-ua4*, an MHC class I allele previously shown to confer a survival advantage in the Seychelles warbler. I found widespread patterns of allele frequency increase within cohorts consistent with the survival effect, but no overall increase in population allele frequency over time. I investigated potential antagonistic reproductive mechanisms, but found no clear evidence for why this allele is not driven towards fixation. Collectively, my results provide an interesting case study of the evolutionary conservation approach, whilst providing insight into the importance of maintaining genetic variation in natural populations.

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Tea, anyone…?

Contents

Author Contributions

At the time of final submission, three of the five data chapters presented in this thesis are published and one is in review. I am lead or joint-lead author on all manuscripts and I have made by far the largest contribution to the work presented in this thesis. Below, I provide a citation for each data chapter, highlight authorship and specify my contributions.

Chapter 2: Spurgin LG*, Wright DJ*, Van der Velde M, Collar NJ, Komdeur J, Burke T & Richardson DS (2014) *Evolutionary Applications* doi: 10.1111/eva.12191.

- DJW role in locating and sampling museum specimens, lab work and drafting manuscript (40%). **joint primary authorship*

Chapter 3: Wright DJ, Spurgin LG, Collar, NJ, Komdeur J, Burke T & Richardson DS (2014) *Molecular Ecology* **23**, 2165-2177.

- DJW role in designing the study, fieldwork and translocation (Frégate), lab work, lead data analyses and drafting manuscript (75%)

Chapter 4: Wright DJ, Shah NJ & Richardson DS (2014) *Conservation Evidence* **11**, 20-24.

- DJW role in translocation and drafting manuscript (70%)

Chapter 5: Wright DJ, Mannarelli ME, Komdeur J, Burke T & Richardson DS (in review: *Behavioral Ecology*)

- DJW role in fieldwork, lab work, lead data analyses and drafting manuscript (70%)

Chapter 6: Wright DJ, Spurgin LG, Dugdale HL, Komdeur J, Burke T & Richardson DS (in prep.)

- DJW role in lab work, lead data analyses and drafting manuscript (65%)

Chapter 1

General introduction

Cousin Island, with Praslin Island in the background

1.1 Challenges in evolutionary and conservation genetics

"…reinforcing the grounds for nature conservation with an evolutionary perspective may help to give conservation a permanence which a utilitarian, and even an ecological grounding, fail to provide in men's minds."

Otto Frankel, 1974

Evolutionary and conservation genetics are both concerned with the ability of populations to evolve in response to environmental change (Reed & Frankham 2001). A central principle of evolutionary biology is that evolutionary change depends on heritable variation. Consequently, the ability of species to evolve is primarily determined by adaptive, or functional, genetic variation (Frankham 2010). Understanding the amount and persistence of this variation within natural populations is a fundamental objective for evolutionary biologists. Conservation genetics involves the application of molecular genetic principles and techniques to the conservation of biodiversity (Frankham 2010). In the mid 20th century, a landmark paper by Frankel (1974) highlighted the need to integrate evolutionary approaches into the understanding and conservation of biodiversity. Frankel's (1974) key message was that maintaining genetic diversity, and thereby conserving the *evolvability* of species in an unpredictable future, should be a central goal for conservation biologists. This amalgamation of the two disciplines is now recognised in the emerging field of evolutionary conservation (Crandall *et al.* 2000; Ferrière *et al.* 2004; Allendorf & Luikart 2007; Carroll & Fox 2008; Höglund 2009).

We are currently witnessing an unprecedented loss of global biodiversity (Pimm *et al.* 1995; Myers & Knoll 2001; Butchart *et al.* 2010). Consequently, there is an increasingly urgent need for evidence-based conservation, underpinned by an understanding of species and ecosystem ecology and evolution. Several authors have identified key challenges in evolutionary conservation genetics and provide guidance for future research. For instance, Pertoldi *et al.* (2007) outline the need to 1) unravel the distribution of variation in functional versus non-coding sequences in natural populations, 2) merge ecological and genetic data when monitoring natural populations for conservation, and 3) collect informative genetic and environmental data sets from natural populations, as well as from preserved specimens. Frankham (2010) similarly highlights the need to 1) gather estimates of quantitative (adaptive) genetic variation in wild species, 2) gather quantitative estimates of the cost of inbreeding in wild populations, 3) directly monitor the change in genetic diversity of selected species over time, and 4) define and test strategies to manage adaptive variation in threatened species.

Focusing research efforts in this way is particularly important in conservation, where financial constraints are often stringent and severe (Groom *et al.* 2006).

For many years, there has been debate about the role and importance of genetic variation in the persistence and viability of populations (Lande 1988; Spielman et al. 2004; Frankham 2005; Pertoldi et al. 2007). Although this debate is largely resolved and the importance of genetic variation widely recognised (McNeely *et al.* 1990; Saccheri *et al.* 1998; Westemeier *et al.* 1998; O'Grady *et al.* 2006), gaining an understanding of the interaction between genetic, demographic, phenotypic and environmental factors in natural populations remains a significant challenge (Purugganan & Gibson 2003; Pertoldi et al. 2007). The principal aim of this thesis is to address this challenge by 1) investigating the evolutionary mechanisms maintaining genetic variation in a wild population and 2) investigating how population history and conservation action impacts this variation.

1.2 Genetic variation

Understanding how genetic variation is distributed within and between individuals and populations has come a long way since the development of the conceptual (Fisher 1930; Wright 1931; Haldane 1932) and mathematical frameworks of population genetics (e.g. Wright 1943; Nei 1973; Nei *et al.* 1975). Technological advances have provided a succession of molecular genetic tools: e.g. allozymes (Hubby & Lewontin 1966), restriction-fragment length polymorphisms (RFLP) and microsatellites (Sunnucks 2000) through to single nucleotide polymorphisms (SNPs) (Morin et al. 2004) and the recent proliferation of next-generation sequencing and genomic techniques (Hawkins *et al.* 2010; Metzker 2010). Genetic variation can be broadly considered either neutral, i.e. not directly subject to natural selection, or adaptive and subject to selection. Which to consider in population genetic studies depends heavily on the questions being addressed, as each highlights different sets of evolutionary issues in need of investigation.

1.2.1. Neutral variation

The 'nearly neutral' theory of molecular evolution holds that the majority of evolutionary change at the molecular level is due to random genetic drift (stochastic allele fixation or loss) and mutation, influenced by gene flow and population size (Kimura 1983). As neutral variation is not directly influenced by the pressures of natural selection, it is exceptionally valuable for inferring both population- and individual-level evolutionary processes. At the population level, analysing neutral variation can reveal patterns of population structure (Wright 1931; Jost

2008; Holsinger & Weir 2009), changes in size such as bottlenecks (Nei *et al.* 1975; Cornuet & Luikart 1996; Garza & Williamson 2001), estimates of effective population size (Maruyama & Kimura 1980; Palstra & Ruzzante 2008; Charlesworth 2009), hybridisation (e.g. Roy *et al.* 1994) and speciation (e.g. Barluenga *et al.* 2006). At the individual level, analysis of neutral variation can reveal, for example, kinship, parentage and inbreeding, and enable the construction of pedigrees (Hamilton 1964; Queller & Goodnight 1989; Hadfield *et al.* 2006; Wang 2011). These are amongst the most important parameters in population genetics (Allendorf & Luikart 2007).

Of the available neutral genetic markers, microsatellites have proven highly popular in population genetic studies (Sunnucks 2000). Microsatellites are short, simple sequences of non-coding DNA that vary in the number of motif repeats between loci and individuals (Litt & Luty 1989; Sunnucks 2000). Their usefulness owes largely to their hypervariability and widespread occurrence throughout genomes and across taxa (Sunnucks 2000; Ellegren 2004). The mutation rate of microsatellites is comparatively rapid and so provides high resolution for studying even short-term genetic processes (Ellegren 2004). However, this can also be problematic in certain instances, as the high mutation rate of these markers can result in homoplasy and subsequent misinterpretation of long-term genetic data (Estoup *et al.* 2002). This highlights a fundamental point in molecular ecology: the importance of selecting the right molecular tools, with the right properties, for the job.

As the use of molecular markers allowed insights into population and individual-level genetic processes, attempts quickly followed to link this genetic variation to components of fitness, i.e. traits under the direct influence of natural selection. These often focused on correlating heterozygosity across suites of microsatellite markers with fitness traits of interest, known as heterozygosity-fitness correlations (HFCs), in studies of inbreeding where pedigree information was absent. Three hypotheses have been proposed to explain such correlations. (1) In 'local effects', the fitness effects are the result of other loci in close linkage disequilibrium (non-random association of loci) with the loci under investigation. (2) In 'general effects', the loci under investigation are distributed genome-wide and thus represent overall effects through identity disequilibrium (non-random association of genotypes; reviewed in Hansson & Westerberg 2002). (3) 'Direct effects' are the result of heterozygote advantage at the very loci under investigation, but this hypothesis is not relevant to HFCs conducted with microsatellites, as such studies assume marker neutrality.

HFCs remain a controversial topic (Chapman *et al.* 2009; Szulkin *et al.* 2010). In brief, they assume that diversity at (an often small) suite of neutral markers accurately estimates genome-wide diversity at adaptive loci (David 1998). Correlations between such measures of genome-wide variation and fitness traits (and therefore adaptive genetic variation) are often weak at best (David 1998; Reed & Frankham 2001; 2003). This is because unless there is high variance in inbreeding across a population, a large set of markers is generally required to accurately measure genome-wide variation (Chapman *et al.* 2009). However, recent work using large marker sets generated by hi-throughput genome sequencing, has confirmed HFCs previously identified by small panels of microsatellites, suggesting that, in at least some instances, the assumption that markers accurately represent genome-wide heterozygosity may be justified (Hoffman *et al.* 2014).

1.2.2 Adaptive, or functional, variation

Adaptive genetic variation can be defined as heritable genetic variation that is directly subject to natural selection, and thus influences an individual's fitness (reviewed in Houle 1992; Frankham 1999; Garcia de Leaniz *et al.* 2007). The study of adaptive genetic variation can be attempted from a "top down", whole-genome approach, or a "bottom up", candidate gene approach. Many genetic traits involved directly in fitness (and thus of conservation and evolutionary interest) are quantitative or polygenic in nature (Frankham 1999). The "top down" approach generally involves scanning all, or at least large parts, of the genome and mapping the relationships between variation at different sites in the genome and particular fitness traits. Over time there have been differing approaches to this including linkage-mapping studies, quantitative-trait-loci (QTL) mapping and, more recently, genome-wide association studies (GWAS), each of which varies in its application of molecular markers, sequence data and bioinformatics approaches (Doerge 2002; Slate 2005; McCarthy *et al.* 2008). Despite studies successfully linking quantitative variation to fitness traits in natural populations (Johnston *et al.* 2011), there are drawbacks to the "top-down" approach. Mapping loci involved directly in fitness traits requires easily quantifiable traits, large sample sizes, and either laboratory-based populations or pedigree information on natural populations (Stinchcombe & Hoekstra 2008). Advances in genomics are continually improving our ability to perform whole-genome scans for quantitative variation, but this suffers from difficulties in uncovering specific functional genes of relevance (Stinchcombe & Hoekstra 2008). Owing to the quantity of bioinformatics involved, these approaches are also susceptible to erroneous statistical results (Amos *et al.* 2010).

The "bottom up" candidate gene approach relies on taking specific genes of known function from other species (e.g. model laboratory organisms) and investigating how variation at these genes affects fitness across individuals within a population (reviewed in Amos *et al.* 2010). This approach has several advantages over "top down" methods as 1) it is readily applicable to natural populations (i.e. those of conservation interest) where pedigrees are often unknown, 2) known genes are easier to target for characterisation and quantification, and 3) by virtue of possessing known functional importance the genes often display clearer links with fitness, which can be helpful when specific gene function in the natural population under investigation is unknown (Amos *et al.* 2010). The key point is that it can elucidate the link between change in genetic variation at a locus, change in a phenotypic trait and change in fitness of the phenotype. The candidate gene approach is limited in that the loci being tested may not be important in the population of study, but the approach still has considerable worth in evolutionary and conservation research in the genomic era (Wilkening *et al.* 2009; Amos *et al.* 2010). Immune-related genes make excellent candidates for this approach as the links between health and evolutionary fitness are well documented across taxa (May & Anderson 1983; Ohlberger *et al.* 2011; McTaggart *et al.* 2012), largely circumventing the risk that the candidate gene may not be important in the study population.

1.3 The major histocompatibility complex

The major histocompatibility complex or MHC is a multigene family central to the vertebrate immune system. These genes code for glycoprotein receptors that bind to antigens, which they then present to cytotoxic T lymphocytes (Klein 1986) and thereby initiate a cascade of immune responses (Hughes & Yeager 1998). Their importance to health is well documented (Meyer & Thomson 2001) and they have become the candidate genes of choice for many evolutionary studies across a range of taxa (Bernatchez & Landry 2003; Sommer 2005; Piertney & Oliver 2006). The MHC contains the most variable functional genes yet described in vertebrates (Garrigan & Hedrick 2003), and attempting to understand how and why this extraordinary diversity exists has made the MHC a paradigm of evolutionary genetic research. Diversity at the MHC is thought to be driven by a combination of three key pathogen-mediated balancing selection mechanisms: overdominance (Doherty & Zinkernagel 1975), negative frequency-dependence (Clarke & Kirby 1966) and fluctuating selection (Hill 1991), along with the influence of sexual selection (Penn & Potts 1999; Spurgin & Richardson 2010) and other mechanisms such as associative balancing complex (ABC) evolution (Van Oosterhout 2009). However, empirical clarification of the relative role of each of these hypotheses in natural systems has proven extremely difficult (Oliver & Piertney 2010; Spurgin & Richardson 2010).

1.3.1 Structure and function

MHC genes can be broadly assigned to two main classes based on the function of their translated molecules (Hughes & Yeager 1998; but see Gruen & Weissman 2001). MHC Class I genes code for proteins that are expressed on the surfaces of all somatic cells except for certain neurons (Klein 1986; Hughes & Yeager 1998). These molecules are heterodimers consisting of an α-heavy transmembrane chain, and possess three extracellular domains (α_1, α_2 & α_3) and a β_2-microglobulin molecule (Figure 1.1; Hughes & Yeager 1998). The peptide-binding region (PBR), responsible for antigen recognition, consists of two α-helices bordering a β-pleated sheet formed from the α_1 and α_2 domains of the heavy chain (Jeffery & Bangham 2000). These molecules are generally involved in detecting intracellular parasites (such as viruses or intracellular bacteria) and initiating non-self recognition by T-cells (Hughes & Yeager 1998; Teacher et al. 2009). In contrast, MHC Class II genes code for molecules that are limited to certain cell types, including B cells, macrophages and dendritic cells (Klein 1986; Ting & Trowsdale 2002). Class II molecules are involved in the immune response to extracellular pathogens (Klein 1986). Structurally, they consist of an α-chain and a β-chain which are both transmembrane proteins (Hill 1991). Similar to class I, there are a total of four extracellular domains, but in class II molecules two extracellular domains are formed by the α-chain (termed α_1 and α_2) and two domains by the β-chain (β1 and β2; Hughes & Yeager 1998). The PBR region of class II, although structurally similar to class I (Jeffery & Bangham 2000), is formed both by the α-chain and by the β-chain (Figure 1.1; Hughes & Yeager 1998).

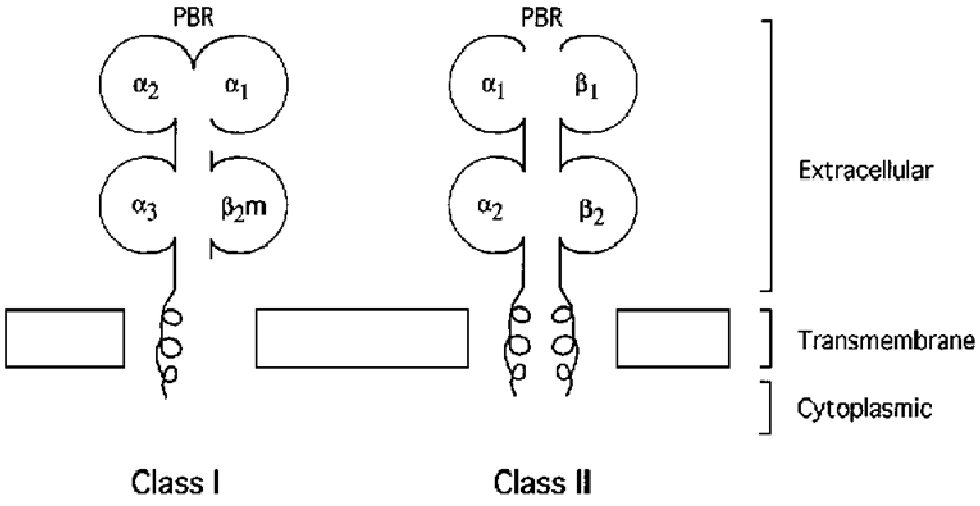

Figure 1.1 Schematic representation of the structure of class I and class II MHC molecules. PBR is the peptide-binding region. *Unmodified figure reproduced from Hughes & Yeager (1998).*

The evolution of the MHC has been debated for many years (reviewed in Nei *et al.* 1997) and three non-exclusive models have been invoked. Under 'divergent evolution', new orthologous genes emerge through gene duplication and diverge gradually, acquiring new functions via mutation. A 'concerted evolution' model was then developed to explain within-species sequence homogeneity of gene families via gene conversion. Finally, a third 'birth-and-death' model was proposed, to explain unusual patterns of evolution in mammalian MHC which could not be explained by either of the preceding models (Holliday 1964; Nei *et al.* 1997; Nei & Rooney 2005). The separation of these models is difficult and they may be non-exclusive (Nei & Rooney 2005). There is also evidence accumulating to suggest that gene conversion acts in avian taxa (Wittzell *et al.* 1999; Burri *et al.* 2010; Spurgin *et al.* 2011).

Although the exact role of the different potential mechanisms in the evolution of the MHC remains unclear, more is known about the organisation of the MHC gene complex, particularly in birds (Hughes & Yeager 1998; Beck *et al.* 1999; Kaufman *et al.* 1999; Hess *et al.* 2000; Westerdahl *et al.* 2000; Hess & Edwards 2002; Westerdahl 2007). Class I and II genes are generally found together in a single gene complex, in a similar layout to mammalian MHC (Beck *et al.* 1999; Hess & Edwards 2002). However, the number of MHC loci varies significantly between taxa (Hess & Edwards 2002) and even between individuals (Eimes *et al.* 2011). The chicken (*Gallus gallus domesticus*) possesses a "minimal essential MHC": comparatively small, gene dense regions with few MHC genes considered indispensible for survival (Kaufman *et al*. 1999). In contrast, passerines appear to possess a highly variable and highly polymorphic MHC, with larger numbers of genes and pseudogenes (e.g. Hess *et al.* 2000; Hess & Edwards 2002; Bonneaud *et al.* 2004; Westerdahl 2007). As gene conversion, and thus homogenisation of genes, is thought to take place in the passerine MHC, ascertaining the relevant locus of a specific allele is difficult without complete sequencing of the MHC region of multiple individuals of a species (Hess & Edwards 2002; Westerdahl 2007).

1.3.2 MHC as a paradigm of adaptive variation

The evolution, function and diversity of the MHC have been the subject of intense research for many years (Potts & Wakeland 1990; Bernatchez & Landry 2003; Sommer 2005; Piertney & Oliver 2006; Spurgin & Richardson 2010). This is due to the association of MHC and key fitness components, such as reproductive success and survival, and the close link between MHC genotype and phenotype (Hughes & Yeager 1998; Milinski 2006; Westerdahl 2007; Oliver & Piertney 2010). Understanding this link between MHC and fitness is important to evolutionary conservation, as it provides insight into how evolutionary processes shape phenotypic traits –

the first step in understanding how to conserve the evolvability of biodiversity. We still cannot fully explain the relative roles of demographic processes, such as bottlenecks or translocations, or deterministic processes, such as selection, and stochastic processes of drift and mutation, in shaping MHC diversity (Oliver *et al.* 2009; Babik 2010; Radwan *et al.* 2010), although many studies are now focusing on addressing gaps in our understanding (e.g. Miller & Lambert 2004; Agudo *et al.* 2011). Factoring this complex interplay of processes into the interpretation of results is a vital step in future MHC research (Oliver & Piertney 2010).

There are limitations in the use of the MHC to study adaptive variation in natural populations. Although unrivalled in their polymorphism, MHC genes are only a single component within a multitude of genes that interact in the vertebrate immune system (Acevedo-Whitehouse & Cunningham 2006). For example, the killer-cell immunoglobulin-like receptors (KIRs) and toll-like receptors (TLRs) are equally significant components of immunocompetence (Belvin & Anderson 1996; Downing *et al.* 2010; Ekblom *et al.* 2010). Interpretation of MHC-dependent effects in population genetic studies must therefore acknowledge that immunity is not determined merely by a single multigene family. Furthermore, evidence that low MHC diversity reduces population viability is somewhat equivocal, as studies report populations surviving despite little or no diversity (Radwan *et al.* 2010). Whether this is due to a publication bias towards successful conservation outcomes remains unknown (Radwan *et al.* 2010). Frankham (2010) similarly argues that research into quantitative variation has been largely *limited* to MHC and that this represents only a "modest proportion" of adaptive variation present in the genome. Notwithstanding these criticisms, the MHC remains an informative, readily quantifiable and challenging puzzle (Bernatchez & Landry 2003; Oliver & Piertney 2010).

1.3.3 Reference strand-mediated conformational analysis (RSCA)

Several methodologies exist for characterising variation at the MHC, including non-PCR based methods, such as RFLP analysis, and PCR-based conformational analyses and next-generation sequencing (Babik 2010). Of these, conformational mutation detection (or 'indirect typing') methods have proved popular due to their universal applicability, economic cost and ability to detect variation at single nucleotide resolution (Babik 2010), although genomic technologies are now undoubtedly superseding these (Metzker 2010). Reference strand-mediated conformational analysis (RSCA) is an indirect typing technique which involves the hybridisation of unknown sequences (allelic variants) to a known fluorescently labelled reference strand (FLR). The resulting heteroduplexes differ in conformation due to annealing mismatches

between the FLR and unknown sequence. When subject to non-denaturing gel electrophoresis, these heteroduplexes exhibit different electrophoretic mobility based on the extent of the mismatch, which can be used to distinguish between sequences on a semi-automated DNA sequencer (Argüello et al. 1998). A disadvantage of this technique is that some prior knowledge (i.e. preliminary screening of variation) of the study system is required for the design of suitable FLRs (Argüello *et al.* 1998; Lenz *et al.* 2009), although it lends itself readily to hi-throughput genotyping, at least in systems with reduced MHC diversity (Richardson *et al.* 2005). Initially developed as a human diagnostic tool, RSCA has been employed across a range of taxa to address diverse questions in evolution, ecology and conservation. For example, Drake *et al.* (2004) applied the RSCA technique to MHC diversity in wild populations of cheetah (*Acinonyx jubatus*), finding evidence of recombination and generation of new allelic variation. RSCA typing has also been employed in MHC-based mate choice studies on semi-natural three-spined stickleback (*Gasterosteus aculeatus*) populations (Eizaguirre et al. 2009). Worley *et al.* (2008) used the technique to characterise the MHC of the red jungle fowl (*Gallus gallus*) and subsequently to investigate MHC-dependent survival (Worley *et al*. 2010). It is the methods of Worley *et al.* (2008) that I utilise in the MHC analyses presented in this thesis.

1.4 Evolutionary conservation of genetic variation

Populations of conservation concern are, by definition, often small in size (Groom *et al.* 2006; Allendorf & Luikart 2007). Size plays a significant role in determining the amount and arrangement of genetic variation in populations and therefore influences long-term evolvability (Fisher 1930; Wright 1931; Haldane 1932). As drift leads to the loss or fixation of alleles through incomplete random sampling in each generation, genetic diversity is expected to erode more rapidly in smaller populations, since drift is more severe the smaller the population size (Wright 1931). Genetic drift and selection can subsequently change allele frequencies between populations (Wright 1922) and, if they are isolated, lead to differentiation and ultimately to speciation (Darwin 1859; Wright 1943; Brekke *et al.* 2011).

Over time, limited size and isolation will cause inbreeding in small populations as individuals become more closely related, even if the population is panmictic (Allendorf & Luikart 2007). This may result in inbreeding depression, which produces two distinct problems: the reduced fitness of individuals due to the expression of accumulated deleterious mutations at loci where the individual is homozygous (Charlesworth & Charlesworth 1987; Crow 1993) and the loss of heterozygote advantage, the relative fitness benefit of being a heterozygote (Charlesworth & Willis 2009). Maintenance of genetic variation is therefore a key concern of small populations,

such as captive or island populations (Hedrick & Kalinowski 2000; Marr *et al.* 2006; Frankham *et al.* 2010). Purging (the removal by selection of the accumulated deleterious mutations over time) may alleviate problems associated with inbreeding in small populations, but its efficiency in natural populations is uncertain (Crnokrak & Barrett 2002) and will depend on the severity of inbreeding depression in the population in question.

The duration and magnitude of changes in population size, namely bottlenecks and expansions, dictate the extent to which population-level processes shape genetic variation (Fisher 1930; Kimura 1983; Ellegren *et al.* 1993; Garza & Williamson 2001). For example, a severe bottleneck of a large, panmictic population will cause a significantly larger loss of variation and more extreme inbreeding depression than observed in a bottleneck of a population maintained at a small size for many generations (Allendorf 1986; Charlesworth & Charlesworth 1987; Crnokrak & Barrett 2002). Another consequence of bottlenecks and expansions is that populations may not exhibit evolutionary responses predicted by contemporary selection or demography, owing to a time-lag effect of historic changes in size (Blumstein 2002). Loss of genetic variation reduces population evolutionary potential, or evolvability, and is therefore a concern for small populations (Frankham *et al.* 1999; Keller & Waller 2002). From an evolutionary conservation perspective, this makes understanding population history important as it influences the way we interpret and conserve evolvability of species and biodiversity (e.g. Grueber & Jamieson 2011). For instance, whether or not to instigate assisted gene flow between small, isolated populations would depend on whether, and how far in the past, they were historically a single large population and the effect that this has had on their genetic variation and population differentiation since then (Edmands 2007).

1.4.1 Comparing neutral and adaptive variation

Considerable research effort has been devoted to understanding the differences in how neutral and adaptive variation respond to the individual- and population-level genetic processes, in both laboratory and natural populations (Ellegren *et al.* 1993; Reed & Frankham 2001; Aguilar *et al.* 2004; Hansson & Richardson 2005; Edmands 2007; Schwensow *et al.* 2007; Landguth & Balkenhol 2012; Oliver & Piertney 2012). As adaptive variation is open to selection, such processes may impact it in a different way, or extent, compared to neutral variation. For example a study on isolated populations of the San Nicolas Island fox (*Urocyon littoralis dickeyi*) found near-monomorphic neutral variation across a range of molecular markers and yet relatively high variation at MHC loci. This functional variation is thought to have persisted as a result of balancing selection, despite a severe bottleneck and subsequent inbreeding

(Aguilar *et al.* 2004). Another study suggests linkage of MHC alleles as a possible mechanism counteracting the effects of drift in maintaining antigen recognition capabilities in insular populations of Egyptian vulture (*Neophron percnopterus*; Agudo *et al.* 2011). Understanding whether selection or drift dominates in shaping adaptive variation under different demographic conditions has become a focal point for evolutionary genetic research (e.g. Seddon & Ellegren 2004; Munguia-Vega *et al.* 2007; Miller *et al.* 2010; Grueber *et al.* 2013).

From a conservation perspective, one of the most relevant examples of the need to incorporate neutral and adaptive genetic considerations and species evolutionary history is in the use of translocation. Translocations are an increasingly common conservation tool (Ewen *et al.* 2012). However, historically success rates have been poor (Griffith *et al.* 1989; Wolf *et al.* 1996), and post-release monitoring of genetic impacts have been inadequate (Fischer & Lindenmayer 2000). Translocations effectively simulate bottlenecks, create founder effects (Jamieson 2011) and typically involve small founder sizes subject to inbreeding and drift, alongside ecological and stochastic risks (Tracy *et al.* 2011; Ewen *et al.* 2012). It is therefore important to understand the comparative impact this action has on both neutral and adaptive genetic variation so that we can plan appropriate conservation action (Groombridge *et al.* 2012). A few studies have compared the impact of translocation on neutral and functional diversity in wild populations, finding that drift generally outweighs selection in shaping both kinds of variation (Miller & Lambert 2004; Sutton *et al.* 2011; Bauer *et al.* 2013; Monzón-Argüello *et al.* 2013). However, the reasons for differences in loss of neutral and functional variation during the process of translocation itself remain unresolved. A clear need exists for studies that compare adaptive and neutral variation in natural populations over time, where environmental variables and demographic history of the study populations are known.

1.5 The Seychelles warbler – a model for evolutionary conservation

Species endemic to islands have long been a draw for evolutionary research (Darwin 1859). Islands are discrete, dynamic, readily quantifiable entities, making evolutionary studies here more tractable than in continental systems (Wallace 1902; Whittaker 1998; Emerson 2002). Island endemic species also generally exist as small populations and are often subject to the considerable genetic impacts of small size as well as being of conservation concern (Frankham 1998; Kier *et al.* 2009; Lee & Jetz 2011). Their small geographic ranges make practical conservation action, such as complete eradication of alien invasive species, realistically achievable (Shah 2001; Nogales *et al.* 2004; Howald *et al.* 2007) and island species have been

subjected to extensive translocation effort (Wolf *et al.* 1996; Jamieson 2011; Ewen *et al.* 2012). Collectively, this makes islands attractive systems for evolutionary conservation research.

The Seychelles warbler (*Acrocephalus sechellensis*) is a small passerine endemic to the islands of the Republic of Seychelles in the western Indian Ocean (Safford & Hawkins 2013; Figure 1.2). Seychelles warblers are predominantly insectivorous, and feed on a range of invertebrates. They inhabit a range of arboreal and scrub (including coastal) habitats, except for open areas of granite glacis and coconut (*Cocos nucifera*) dominated forest (Figure 1.3; Komdeur 1991). Historically, the Seychelles warbler probably existed on several islands within the archipelago, although records of its distribution when the islands were first settled (1770s) are limited and unclear (Komdeur 1991; Chapter 2). As a result of human colonisation, alien predators and intensive agriculture, particularly coconut plantations, were introduced that led to the species decline. By the middle of last century, reports suggested that the population comprised only 26-50 birds on Cousin Island (4°20'S, 55°40'E, 0.29 km²; Vesey-Fitzgerald 1940; Crook 1960; Chapter 2). This island was purchased in 1968 by a consortium led by the International Council for Bird Preservation, now BirdLife International, and was then subjected to an intensive programme of habitat restoration and conservation. The Cousin island population of the warbler rapidly recovered, and reached a stable carrying capacity of *ca.* 320 adult birds by 1982 (Brouwer *et al.* 2007). Since then, four translocations of warblers have occurred to ensure continued population growth and expansion (Richardson 2001): 29 birds were translocated to both Aride (4°12'S, 55°40'E, 0.68 km²) and Cousine (4°21'S, 55°39'E, 0.25 km²) in 1988 and 1990, respectively (Komdeur 1994); 58 birds were translocated to Denis (3°48'S, 55°40'E, 1.42 km², Richardson *et al.* 2006) in 2004; and 59 birds to Frégate (4°35'S, 55°56'E, 2.19 km², Chapters 3 & 4) in 2011. There is virtually no inter-island dispersal (0.1% of known birds), as although the species possesses adequate physiology for sustained flight, it appears behaviourally averse to crossing open bodies of water (Komdeur *et al.* 2004).

Seychelles warblers are facultative cooperative breeders (Komdeur 1991). Breeding birds form long-term pair bonds and defend a strictly defined territory year-round (Komdeur 1992). Owing to habitat saturation and subsequent lack of breeding opportunities, some individuals of either sex delay independent breeding and become subordinates within a territory, often their natal territory (Komdeur 1991). In any given breeding attempt some, but not all, of these subordinates may act as helpers and incubate eggs (females only) or provision chicks (males and females) in the dominant birds' nest (Komdeur 1991). In any given breeding season, around 44% of subordinate females gain maternity by laying an egg in the dominant females'

nests (Richardson *et al.* 2001). Subordinate males rarely gain parentage within or outside the group, despite the fact that high levels of extra-pair paternity (EPP) occur with around 40% of all offspring being fathered by males from outside the natal territory (Richardson *et al.* 2001). Although it may take many years to gain a dominant breeding position, once established most dominant birds retain this position until their death (Komdeur 1991). However, around 14% of breeding females are deposed by subordinate females, and *ca.* 68% of these actually remain as subordinate 'grandparent' helpers (Richardson *et al.* 2007). There is typically one clutch per breeding season, which consists of a single egg, although 2 or 3 eggs can occur, particularly in cases of joint-mating by subordinate females (Richardson *et al.* 2001). Although Seychelles warblers may breed throughout the year, there are two peaks in breeding activity: a major peak from June to September and a smaller one from November to March (Komdeur 1991). The evolution and dynamics of this breeding system have been the focus of considerable research, for example providing evidence for biased sex allocation, promiscuity and direct and indirect benefits of cooperative breeding (Komdeur 1996; Richardson *et al.* 2001; Richardson *et al.* 2002; Richardson *et al.* 2003; Van de Crommenacker *et al.* 2004; Eikenaar *et al.* 2007; Komdeur *et al.* 2007; Richardson *et al.* 2007).

Intensive monitoring and study of the Seychelles warbler has been underway since the late 1980s (Komdeur 1991). Colour ringing and blood sampling have been conducted on an annual to biannual basis on Cousin since 1993, and more intensely since 1997 (over 96% of the population ringed since 1997, Richardson *et al.* 2001). Periodical sampling and monitoring have also been carried out on all the other islands. Adult Seychelles warblers have no natural predators, although Seychelles fodies (*Foudia sechellarum*), skinks (*Mabuya* spp.) and crabs (*Ocypode* spp. and *Coenobita* spp.) prey on eggs and nestlings (Komdeur 1991; Veen *et al.* 2000). As there is virtually no inter-island dispersal (Komdeur *et al.* 2004) and little extrinsic mortality, it has been possible to collate data over the entire lifetime of the majority of birds within the population. Components of individual fitness including fecundity, survival, parasite burden, behaviour, territory acquisition and territory quality have been quantified over many years, and this has enabled a wide variety of questions concerning evolution, ecology and conservation to be addressed. The system has already demonstrated its potential in diverse research areas such as inbreeding and inbreeding avoidance (Richardson et al. 2004), natal dispersal (Eikenaar *et al.* 2008), ecotoxicology (Van de Crommenacker *et al.* 2011), habitat restoration (Komdeur & Pels 2005), parasite-mediated selection (Hutchings 2009) and senescence (Hammers *et al.* 2012; Barrett *et al.* 2013) as well as those relating to the cooperative breeding system already mentioned.

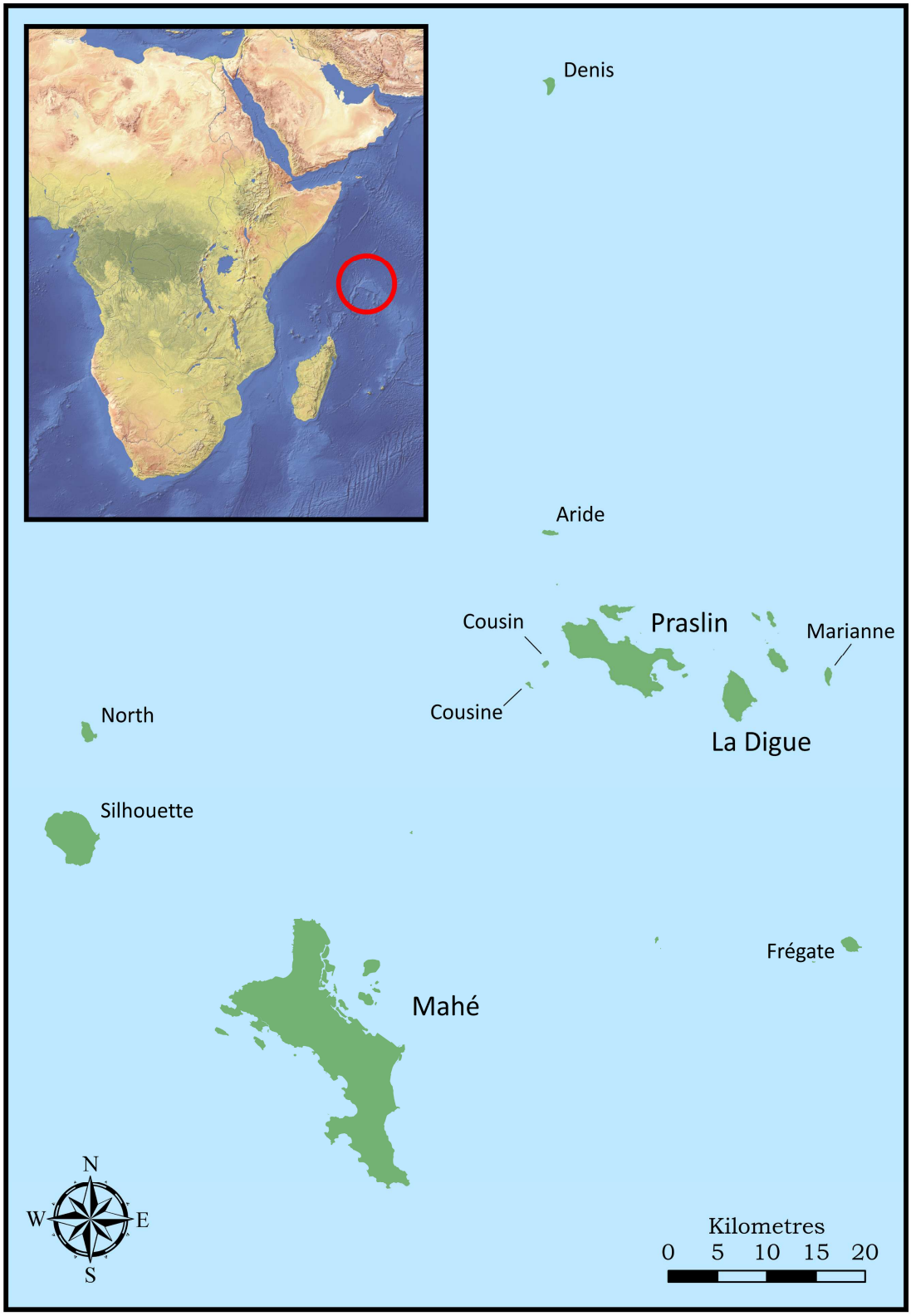

Figure 1.2 Map of the Inner Seychelles islands (main) with global position (inset). Seychelles warbler populations exist on Cousin, Cousine, Aride, Denis and Frégate. Historic records suggest a recently extinct population existed on Marianne. *Map produced using open source FAO data by D. Wright and E. Warren-Thomas.*

Figure 1.3 A) an adult Seychelles warbler (*Acrocephalus sechellensis*), **B)** ideal habitat of mixed native woodland (primarily *Pisonia grandis*, *Morinda citrifolia* and *Ochrosia oppositifolia*) and **C)** unsuitable habitat dominated by coconut (*Cocos nucifera*)

Several key attributes make the Seychelles warbler project useful in addressing questions in evolutionary conservation. The recent history of this species is relatively well documented and a number of museum specimens exist which can be used to help infer historic genetic and demographic population parameters (Higuchi *et al.* 1984; Bouzat *et al.* 1998; Groombridge *et al.* 2009; Chapter 2). The species has also been subject to intense conservation effort with multiple translocations of different founder sizes allowing exploration of the impact of conservation intervention on genetic diversity. Neutral variation is readily quantifiable, with a suite of microsatellites already developed for earlier parentage studies (Richardson *et al.* 2000; Richardson *et al.* 2001, Chapter 2). The MHC has also been characterised and there is evidence to suggest balancing selection is acting on MHC Class I, as diversity has been maintained in spite of the bottleneck (Richardson & Westerdahl 2003; Hansson & Richardson 2005; Hutchings 2009). The Seychelles warbler also experiences a depauperate parasite fauna, with no evidence of any viral infections, only a single malaria strain *GRW-1* and no gastrointestinal parasites found (to date), making host-parasite complexity more tractable (Hutchings 2009).

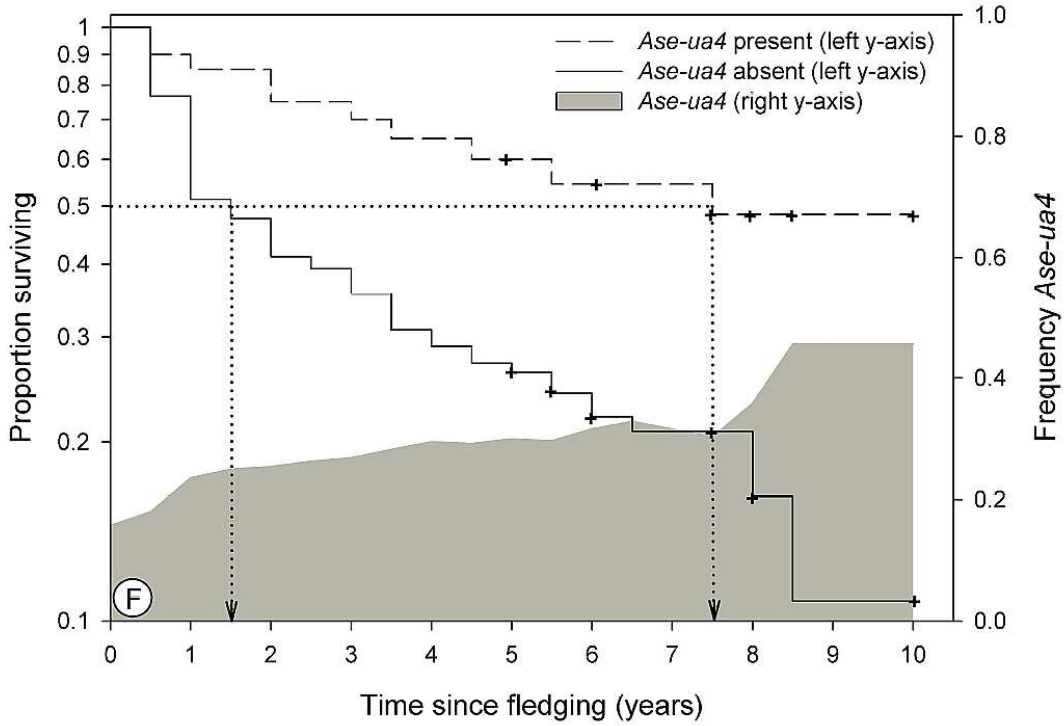

Figure 1.4 The survival advantage associated with a single MHC class I allele, *Ase-ua4,* in a cohort of Seychelles warblers on Cousin Island. Its effect on median life expectancy is shown as survival curves, along with the allele frequency increase shaded in grey. *Unmodified figure reproduced from Brouwer et al. (2010).*

Interactions between MHC and extra-pair paternity (Richardson *et al.* 2005) and survival have also been documented (Figure 1.4; Brouwer *et al.* 2010), providing further research opportunity. Collectively, the Seychelles warbler research project provides an ideal platform in which to explore how historic and contemporary processes shape neutral and adaptive genetic variation over a large temporal scale, in a natural, unmanaged system with the genetic attributes of small populations subject to intense conservation effort.

1.6 Thesis outline

In this thesis, I investigate how evolutionary forces and conservation action shapes neutral and functional genetic variation within and among populations of the Seychelles warbler. In chapter 2, I study microsatellite diversity in museum-sourced DNA and use these data in conjunction with historic records to reconstruct the species bottleneck and provide evolutionary context to contemporary levels of genetic variation. In chapter 3, I investigate how the four translocations of Seychelles warblers have impacted on both MHC and microsatellite diversity in both the original and translocated populations, and discuss how this helps inform future conservation of the Seychelles warbler and other endangered species. In chapter 4, I present a technical report of the fourth translocation to Frégate Island, in which I played a key role during my PhD, detailing practicalities and monitoring outcomes as a tool for conservation practitioners. In chapter 5, I describe how I used the two most recent translocations as experiments in which to investigate MHC-based social mate choice and whether this provides an evolutionary mechanism helping to maintain MHC diversity. In chapter 6, I investigate the long-term population consequences of a previously detected association between a specific MHC allele and individual survival (Figure 1.4), and assess how different potentially antagonistic evolutionary forces may act to maintain variation in this population. Finally, in chapter 7 I discuss my findings from chapters 2 – 6 in context of evolutionary conservation and suggest possible directions for future research.

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Chapter 2

Museum DNA reveals the demographic history of the endangered Seychelles warbler

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Three Seychelles warbler specimens collected 1877-1888

2.1 Abstract

The importance of evolutionary conservation – how understanding evolutionary forces can help guide conservation decisions – is now widely recognised. However, the historical demography of many endangered species is unknown, despite the fact that this can have important implications for contemporary ecological processes, and for extinction risk. Here we reconstruct the population history of the Seychelles warbler (*Acrocephalus sechellensis*) – an ecological model species. By the 1960s this species was on the brink of extinction, but its previous history is unknown. We used DNA samples from contemporary and museum specimens spanning 140 years to reconstruct bottleneck history. We found a 25% reduction in genetic diversity between museum and contemporary populations, and strong genetic structure. Simulations indicate that the Seychelles warbler was bottlenecked from a large population, with an ancestral N_e of several thousand falling to <50 within the last century. Such a rapid decline, due to anthropogenic factors, has important implications for extinction risk in the Seychelles warbler, and our results will inform conservation practices. Reconstructing the population history of this species also allows us to better understand patterns of genetic diversity, inbreeding and promiscuity in the contemporary populations. Our approaches can be applied across species to test ecological hypotheses and inform conservation.

2.2 Introduction

Evolutionary processes are often overlooked by biologists and policy makers interested in conserving endangered species. This is a problem, as understanding the demographic history of populations, and so the evolutionary pressures that they may have faced, is of conservation importance. High levels of inbreeding in small populations can increase homozygosity, and thus the expression of deleterious recessive alleles, with negative fitness consequences (inbreeding depression; Charlesworth & Charlesworth 1987). A second problem in small populations is the loss of allelic diversity at functional genes, which can compromise the ability of a population to adapt to new or changing environments (loss of evolutionary potential; Soulé 1985). Inbreeding depression and loss of evolutionary potential will almost certainly increase the risk of extinction of populations, and by proxy, species (Frankham 1995; Saccheri *et al.* 1998). One important factor to consider is how long populations have been small: in populations that have experienced continuous, long-term exposure to inbreeding (e.g. small island populations), genetic load can be purged (Crnokrak & Barrett 2002). Although the efficiency with which purging in natural populations reduces genetic load remains uncertain, populations that have been small for a long time may be of less conservation concern than populations that have undergone recent, drastic reductions in population size (Crnokrak & Barrett 2002).

Piecing together the history of wild populations is also of broader biological interest as it helps researchers make sense of present-day behavioural and ecological processes. Historical population bottlenecks, in particular, can affect patterns of individual survival, reproduction and mating behaviour for many subsequent generations, even if the population recovers (Keller *et al.* 1994; Bijlsma *et al.* 2000). Some populations may therefore not exhibit the evolutionary responses predicted based on their contemporary demography and selection pressures, due to time-lag effects of historical population declines, expansions or isolation events (Blumstein 2002).The effects of demographic history on these parameters will depend on the timing, extent and duration of the bottleneck (Miller & Hedrick 1991; Briskie & Mackintosh 2004). However, in most cases detailed data on population history are not available, especially for wild species in which historical bottlenecks have rarely been observed or accurately documented.

Genetic markers can be used to provide insights into individual and population-level processes that are not directly observable. At neutral loci, changes in population size leave signatures on patterns of population-level genetic variation, and a number of methods of detecting

bottlenecks from genetic data have been developed (Cornuet & Luikart 1996; Garza & Williamson 2001). Recent developments in analytical methods, particularly simulation-based approaches, have enabled researchers to use genetic data to make increasingly complex and detailed inferences about demographic history (Hoban *et al.* 2012). An especially promising approach is approximate Bayesian computation (ABC) which has been used to infer the timing, duration and severity of bottlenecks, and to reconstruct pre-bottleneck ancestral population sizes from present-day data (Hoffman *et al.* 2011; Fontaine *et al.* 2012). However, one problem with using present-day DNA samples to study population history is that inferences are still indirect, with the possibility of introducing errors when inferring process from pattern. Different demographic histories can leave similar genetic signatures in contemporary populations and can also mask one another, meaning key demographic events can be misinterpreted or not detected (Lavery *et al.* 1996; Schoville *et al.* 2012). The study of DNA from museum specimens, archaeological finds and fossil remains, can address these issues (Hofreiter *et al.* 2001; Pääbo *et al.* 2004). By comparing historical and contemporary DNA sequences or markers, studies have been able to assess directly how genetic diversity changes through time in non-model organisms. Most of these studies have been restricted to one or a few loci, such as short regions of mitochondrial DNA (Shapiro *et al.* 2004; Calvignac *et al.* 2008), microsatellites (Bouzat *et al.* 1998; Tucker *et al.* 2012) or adaptive loci such as the MHC (Smulders *et al.* 2003). Museum DNA may be especially powerful when used in conjunction with a coalescence-based simulation approaches such as ABC (Chan *et al.* 2006). From a conservation perspective, much of the strength of this approach lies in its use in linking historic and contemporary population changes to anthropogenic impacts, or otherwise, and subsequently directing conservation efforts.

Here, we examine how genetic diversity has changed over a period of 140 years in the Seychelles warbler (*Acrocephalus sechellensis*), a small passerine endemic to the Seychelles archipelago in the Indian Ocean (Figure 2.1). This species was reduced to a single population of reportedly fewer than 30 individuals on the tiny island of Cousin (4°20'S, 55°40'E, 0.29 km²) in the 1960s (Penny 1967; Loustau-Lalanne 1968), and has been of great conservation interest (Penny 1967; Loustau-Lalanne 1968; Komdeur 1994; Komdeur & Pels 2005). Moreover, the Seychelles warbler is a long-term, ecological and evolutionary model system (Komdeur 1992; Richardson *et al.* 2003; Barrett *et al.* 2013), making it an excellent candidate for studying population history. The warbler was confined to a restricted range (two predator-free islands) in the late 19th Century (see Methods) and it is unknown whether the species was ever more widespread across the Seychelles, although it is assumed that it would have been so before the

introduction of predators such as rats (*Rattus* spp.) and cats (*Felis catus*; Collar & Stuart 1985). Relatively low levels of genetic diversity have been observed in the warbler at neutral and functional loci, and this has been linked to fitness (Richardson *et al.* 2005; Brouwer *et al.* 2007; Brouwer *et al.* 2010). Despite this, the roles of natural and anthropogenic factors in shaping the patterns of genetic diversity in this species remain unknown. We first use DNA extracted from museum specimens to compare microsatellite diversity in the historical population with the contemporary population, allowing us to calculate how much genetic diversity has been lost over time. We then use a Bayesian approach, informed by available knowledge of the warbler's demographic history, to estimate ancestral population size and determine the timing and severity of the bottleneck. We then discuss how natural and anthropogenic factors have shaped patterns of genetic diversity in the Seychelles warbler, as well as the broader implications of using our methods and results for evolutionary and conservation biology.

2.3 Materials and methods

2.3.1 Study species and sampling

The demographic history of the Seychelles warbler is outlined in Figure 2.1. The species was first described in 1878 by Oustalet (1878) from the island of Marianne (96 ha), and in the same account was said by Lantz to be "rare on Île Cousine". Subsequent studies found the warbler on Cousin, but not Cousine, and Lantz's account was presumed to be a mistake (Vesey-Fitzgerald 1940). By 1938 the warbler was extinct on Marianne, and Vesey-Fitzgerald (1940) remarked that it "must be the rarest [bird] in the world". Expeditions to Cousin in 1959, 1965, 1967 and 1968 documented 30, 50, 26 and 50 individuals, respectively (Penny 1967; Loustau-Lalanne 1968). However, birds were not uniquely ringed during these trips, so these estimates of population size are unlikely to have been very precise. In 1967 Cousin was designated as a nature reserve, and efforts began to increase the populations of native bird species (Penny 1967). Habitat restoration, consisting of the removal of coconut palms (*Cocos nucifera*) and succession of natural pisonia (*Pisonia grandis*) dominated woodland, was successful and the Cousin warbler population quickly recovered; since the 1980s it has been at a carrying capacity of *ca.* 320 adults (Brouwer *et al.* 2009). Between 1987 and 2011, four new warbler populations were successfully established by translocation to the islands of Aride, Cousine, Denis and Frégate (Komdeur 1994; Richardson *et al.* 2006; Chapters 3 & 4).

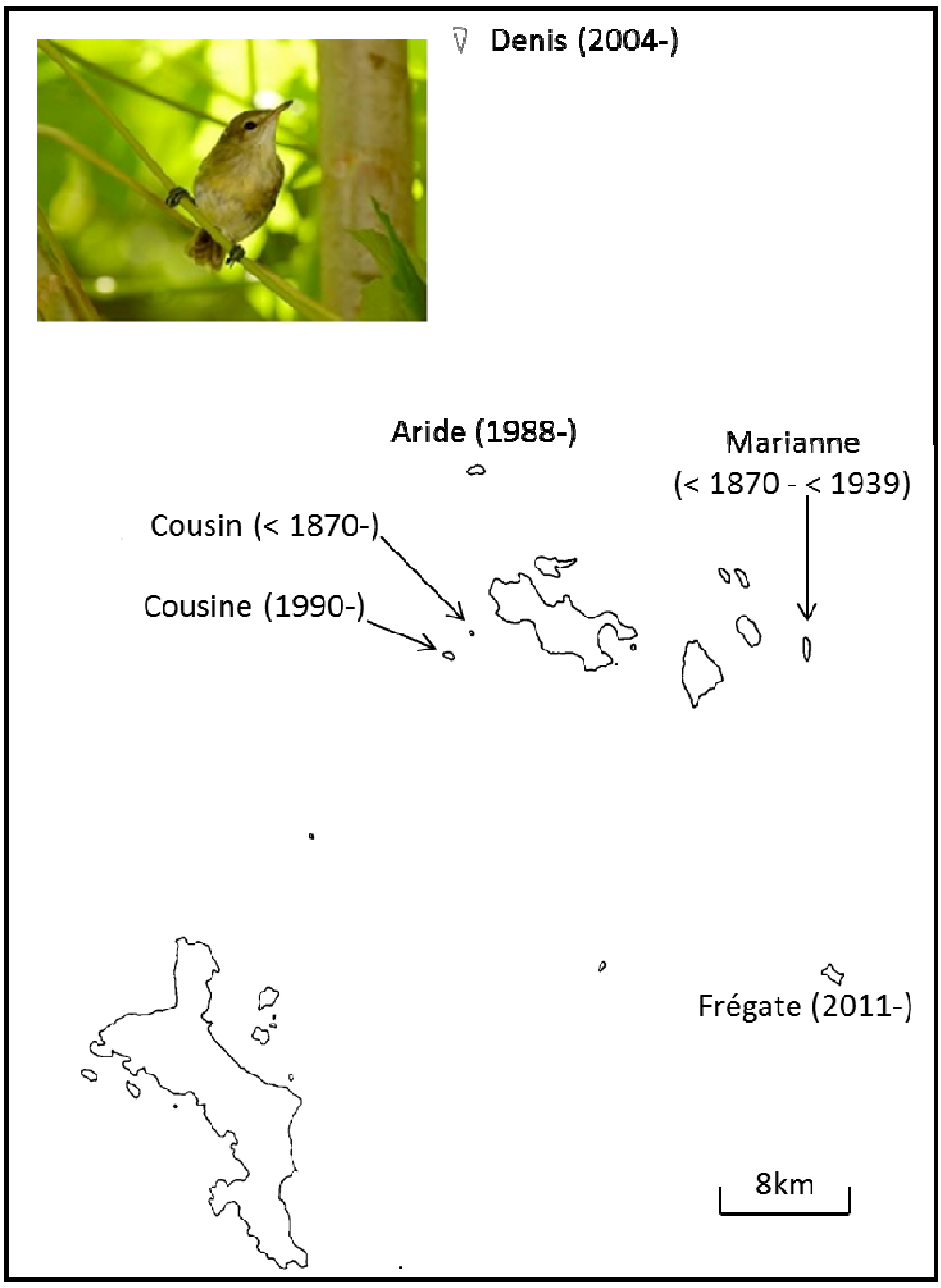

Figure 2.1 Population history of the Seychelles warbler (pictured inset). Dates represent first dates that Seychelles warblers were present on individual islands, and the last known date on Marianne, where the warbler was known to exist but is now extinct. Note that populations on Cousine, Aride, Denis and Frégate were established by translocations.

Historical samples were obtained from all known Seychelles warbler museum specimens, collected from Cousin (n = 19) and Marianne (n = 7) in 1876–1940 (Supplementary Table S2.1). Although the temporal range of sampling of the museum specimens was wide, structure analyses suggested that they grouped into two populations (see Results), enabling us to group them for population genetic analyses. A small (*ca.* 1.5 x 1.5 x 3.0 mm) piece of skin was excised

from the ventral surface of the foot (Figure 2.2) and stored at room temperature in a sterile microfuge tube. Contemporary samples were collected as part of an intensive, long-term study of Seychelles warblers on Cousin Island (Komdeur 1991; Brouwer *et al.* 2010). Since 1988 the entire population has been extensively monitored, often in both the main (June–September) and minor (November–March) breeding seasons each year, during which birds are routinely caught with mist nets and audio lures. A blood sample (*ca.* 25 µl) was collected from each bird by brachial venipuncture and stored at room temperature in a screw-topped microfuge tube containing 1.5 ml absolute ethanol. Each bird was fitted with a unique combination of three colour rings and a metal British Trust for Ornithology (BTO) ring. Over 96% of adult birds on Cousin have been ringed since 1997 (Richardson *et al.* 2001) and a representative sampling of the population was achieved in each year. For the present analysis, 50 samples were randomly chosen from 1997 and 2011 (out of 160 and 197 samples available from that year, respectively) to provide two temporally distinct contemporary population samples for comparison with the historical data.

Figure 2.2 Seychelles warbler museum specimen with a red arrow highlighting the location of the incision on the proximal phalanx to minimise specimen damage. Image taken post-sampling.

2.3.2 Molecular methods

Contemporary DNA was extracted using a salt extraction protocol (Richardson *et al.* 2001). DNA was extracted from the museum samples using a Qiagen DNeasy tissue kit (Qiagen, Crawley, UK) under the manufacturer's instructions, with the following alterations: (1) each sample was finely chopped in a small volume of ATL buffer prior to digestion with proteinase K; (2) 20 µl 1 M DTT (Dithiothreitol, Sigma-Aldrich, UK) was added at incubation; and (3) 1 µl carrier RNA (Qiagen, final concentration = 20 µg/ml) was added during the precipitation phase (Freed & Cann 2006). To reduce the risk of contamination, extractions were performed in separate batches of four, with the incorporation of a negative control at the extraction and PCR stages. All DNA extractions were performed in a laminar flow cabinet in a 'clean room' located separate from the main laboratory. No passerine DNA had previously been processed in this facility. All equipment was isolated exclusively for museum sample extraction, regularly cleaned with industrial methylated spirits (IMS), UV sterilised and all materials were autoclaved where appropriate.

The potential for amplification of each museum DNA sample was tested by molecular sexing using the Z-002D marker set (Dawson 2008). Each PCR included 2 µl Qiagen PCR multiplex master mix, 1 µl primer mix (at a final concentration of 0.2 µM) and 1 µl DNA. The PCR cycling conditions were 15 minutes at 95°C, followed by 45 cycles of 30 seconds at 90°C, 1 minute 30 seconds at 56°C and 1 minute 30 seconds at 72°C. All pre-PCR work was carried out in the 'clean room'. Each contemporary sample was molecularly sexed and genotyped at 30 polymorphic microsatellite loci combined into four multiplexes (Supplementary Tables S2.2, S2.3). PCR amplification of the contemporary samples was performed in 10-µl volumes containing 20–50 ng of template DNA, using a Qiagen Multiplex PCR Kit and the manufacturer's protocol. The PCR program used was as follows: 95°C for 15 min, followed by 30 or 35 (for the museum specimens) cycles of 94°C for 30s, annealing temperature (T_a; multiplex-specific, Supplementary Table S2.2) for 90 s and 72°C for 60 s, followed by 60°C for 30 min. All PCR products were separated on a 48-well capillary ABI 3730 DNA analyser and allele sizes assigned using GeneMapper 4.0 software. All samples were genotyped at least twice to assess repeatability, and new alleles (i.e. those that have not been found in the routine genotyping of contemporary individuals during the long-term project) were only confirmed when observed in both reactions. Twelve of the 30 markers were successfully genotyped using the partially fragmented DNA extracted from the museum samples (Supplementary Table S2.4), so only the corresponding loci from the contemporary samples were included in the dataset used for comparison.

2.3.3 Statistical analyses

Unless stated otherwise, all plots and statistics were generated in R version 2.14.1 (R Development Core Team 2012). For each locus and population (Marianne museum; Cousin museum, 1997 and 2011), we calculated observed and expected heterozygosity and tested for deviations from Hardy–Weinberg equilibrium (HWE) using GENEPOP version 4.0.10 (Raymond & Rousset 1995). Null allele estimates were calculated in CERVUS version 3.0.3 (Marshall *et al.* 1998). Allelic richness and number of private alleles in each population were calculated after controlling for differences in sample size, using a rarefaction approach implemented in HPRare (Kalinowski 2005).

To examine patterns of genetic structure across populations, we calculated pairwise F_{ST}, using Slatkin's (1995) transformation. For comparison, we also calculated Jost's (2008) D_{EST} in SMOGD version 1.2.5 (Crawford 2010). Additionally we looked for evidence of genetic structure using a Bayesian clustering algorithm implemented in the program STRUCTURE (Pritchard *et al.* 2000). We used a model allowing admixture and correlated gene frequencies, and included sampling locations as prior information to deal with the low sample size on Marianne and with potentially subtle structure within our samples (Kalinowski 2011; Porras-Hurtado *et al.* 2013). We carried out four independent runs for each value of K = 1 to 5. For each run, we used 500,000 steps, with a burn-in of 10,000 steps. The value with the highest mean 'log probability of data' was considered the most likely number of clusters. We also inferred the most likely numbers of clusters using the method of Evanno *et al.* (2005). Finally, we carried out a principal components analysis of the microsatellite data using the ADEGENET package in R (Jombart 2008).

We used two approaches based on summary statistics to detect whether the warbler populations had undergone genetic bottlenecks. We first tested for heterozygosity excess, which occurs owing to the loss of rare alleles shortly after bottlenecks (Cornuet & Luikart 1996), using the program BOTTLENECK (Piry *et al.* 1999). We used a two-phase mutation model, and ran the analysis three times, with the percentage of stepwise mutations set at 95%, 90% and 80%, respectively. The probability of heterozygosity excess was calculated using Wilcoxon rank-sum tests. We also calculated Garza and Williamson's (2001) index (M), by dividing the number of alleles in a population (k) by the range in allele size (r).

We used the program DIY-ABC v2.0 (Cornuet *et al.* 2008; Cornuet *et al.* 2014) to estimate the timing and severity of the Seychelles warbler bottleneck. Because of the low sample size on

Marianne, we excluded this population, and focused on the temporal samples from Cousin. We constructed a bottleneck scenario, using the following priors: the pre-bottleneck N_e, post-bottleneck N_e and the timing of the bottleneck (Table 2.1). For comparison, we tested the bottleneck scenario against a null model, which simulated a constant N_e on Cousin over time (Table 2.1). We assumed a generation time of four years, which corresponds to the median age of successful breeders (M. Hammers, unpublished data), and dated the museum population at 26 generations before the 2011 samples (the mid-point of the range of museum sample dates). All priors were given uniform distributions, informed where possible by knowledge of the Seychelles warbler population history. The microsatellite loci were assumed to follow a stepwise mutation model, with mean mutation rate drawn from a uniform prior with minimum and maximum values set at 10^{-3} and 10^{-5}, respectively, with individual locus mutation rate drawn from a gamma distribution (mean = mean mutation rate and shape = 2). For each scenario we simulated 1 million datasets. As summary statistics we used the mean number of alleles per locus, mean gene diversity and mean Garza-Williamson *M* index within each population, as well as pairwise F_{ST} across each pair of populations. The posterior probability of scenarios was estimated by i) taking the 500 simulated datasets closest to the observed dataset and calculating the proportion that belong to each scenario (direct estimate), and ii) performing a logistic regression on the closest 1% of datasets to the observed data (Cornuet *et al.* 2008). We evaluated the posterior distribution of estimates by performing a regression on the closest 1% of logit-transformed datasets, and evaluated bias and precision of each parameter by calculating mean relative bias and relative mean square error using the standard procedures in DIY-ABC.

Table 2.1 Demographic scenarios, priors and posterior estimates used in approximate Bayesian computation (ABC) analyses of the Cousin Seychelles warbler population. Time is in generations (generation time = four years), CI = credible intervals, Bias = mean relative bias and RMSE = relative mean square error (Cornuet *et al.* 2008).

Parameter	Prior	Posterior estimates		Confidence in parameter estimation	
		Median	95% CI	Bias	RMSE
Scenario 1(bottleneck)					
N_e (contemporary)	1–100	46	29–75	0.095	0.434
N_e (ancestral)	1–100,000	6,900	2,400–9,700	-0.032	0.585
Time (bottleneck)	5–500	55	33-64	0.176	0.498

For comparison, we also estimated N_e for the museum (Cousin) and contemporary populations using the Bayesian approach implemented in ONeSAMP version 1.2 (Tallmon *et al.* 2008), with prior minimum values set to 1 and maximum values set to 10,000 for the museum, and 320 for the contemporary population (current census size), respectively. To check how sensitive these estimates were to priors, we re-ran ONeSAMP for the contemporary population using the same, wide priors as for the museum population (1-10,000).

2.4 Results

Of the 26 museum samples, 23 were successfully genotyped at 10 or more of the 12 loci. One sample from Marianne was genotyped at only 3 loci (museum ID 1876-574; Supplementary Table S2.1) so was excluded from further analysis. Two were genotyped at six loci (sample IDs USNM 119753 and AMNH 265502; Supplementary Table S2.1); population-level analyses carried out both with and without these two samples yielded the same results (data not shown), so they were retained, leaving samples sizes of 19 and 6 for Cousin and Marianne, respectively, and a total sample size of 126 individuals when the 1997 and 2011 populations were included. The genotyping error rate in the museum specimens was 2.5%. Observed and expected heterozygosity for each locus and population, along with results of tests for deviations from HWE and null allele frequency estimates, are given in Supplementary Table S2.4.

Table 2.2 Pair-wise F_{ST} (below diagonal) and D_{EST} (above diagonal) in museum and contemporary Seychelles warbler populations. Non-significant values ($P > 0.05$) are highlighted in bold.

	Marianne (M)	Cousin (M)	Cousin (97)	Cousin (11)
Marianne (M)	--	0.09	0.22	0.18
Cousin (M)	0.13	--	0.07	0.05
Cousin (1997)	0.28	0.12	--	**<0.001**
Cousin (2011)	0.27	0.11	**0.002**	--

Pairwise F_{ST} estimates between museum and contemporary samples were moderately large and highly significant (all $F_{ST} > 0.1$, $P < 0.001$; Table 2.2), but there was no significant differentiation between the 1997 and 2011 populations ($F_{ST} = 0.001$, $P = 0.2$; Table 2.2). A PCA suggested that the Cousin and Marianne museum samples formed two separate clusters, with

the contemporary samples forming a separate third cluster (Figure 2.3). STRUCTURE analyses indicated three and two genetic clusters using the log likelihood and Δ*K* methods respectively, which corresponded closely to the results from the PCA (Supplementary Figure S2.1). The only difference between the PCA and STRUCTURE analyses was that STRUCTURE, when *K* = 2, grouped the Marianne samples with the Cousin museum samples (Supplementary Figure S2.1) – however, this is most likely the result of the low sample size on Marianne (Kalinowski 2011).

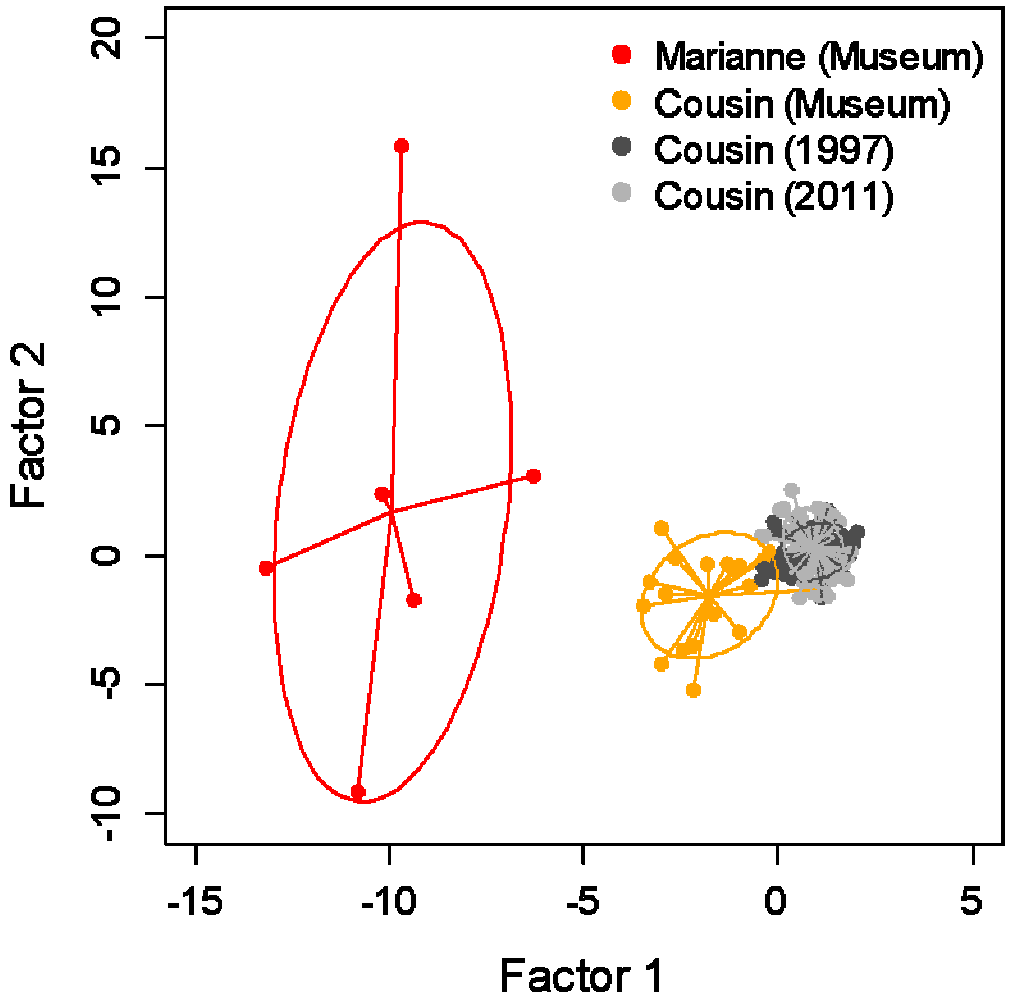

Figure 2.3 Principal components analysis of 126 Seychelles warbler samples, using 12 microsatellite loci. Each point represents an individual, and ellipses represent 95% confidence limits for population-level groups.

In going from the museum to the contemporary populations, there was a 25% loss of allelic richness and a 19% loss of heterozygosity (Figure 2.4). There was significant heterozygosity excess in the Cousin museum population (P = 0.001-0.004, depending on the proportion of stepwise mutations assumed) and in both the 1997 and 2011 populations (P < 0.001 for both years, regardless of the mutation model). There was no evidence for heterozygosity excess in

the Marianne museum populations (*P* = 0.09-0.27), but the number of individuals (n = 6) is lower than the recommended minimum sample size for this analysis (n = 10) and this result must therefore be treated with caution. Mean (± SE) Garza and Williamson's *M* ratio was 0.72 ± 0.06 for the Marianne museum population, 0.81 ± 0.05 for the Cousin museum population, and 0.66 ± 0.05 for both the 1997 and 2011 Cousin populations (M <0.68 is indicative of a bottleneck; Garza & Williamson 2001). As *M* is expected to increase with sample size (Garza & Williamson 2001), these results are conservative. Together, these bottleneck tests indicate that a bottleneck may have already occurred when the museum samples were obtained from Cousin, with strong evidence for a bottleneck in the contemporary samples.

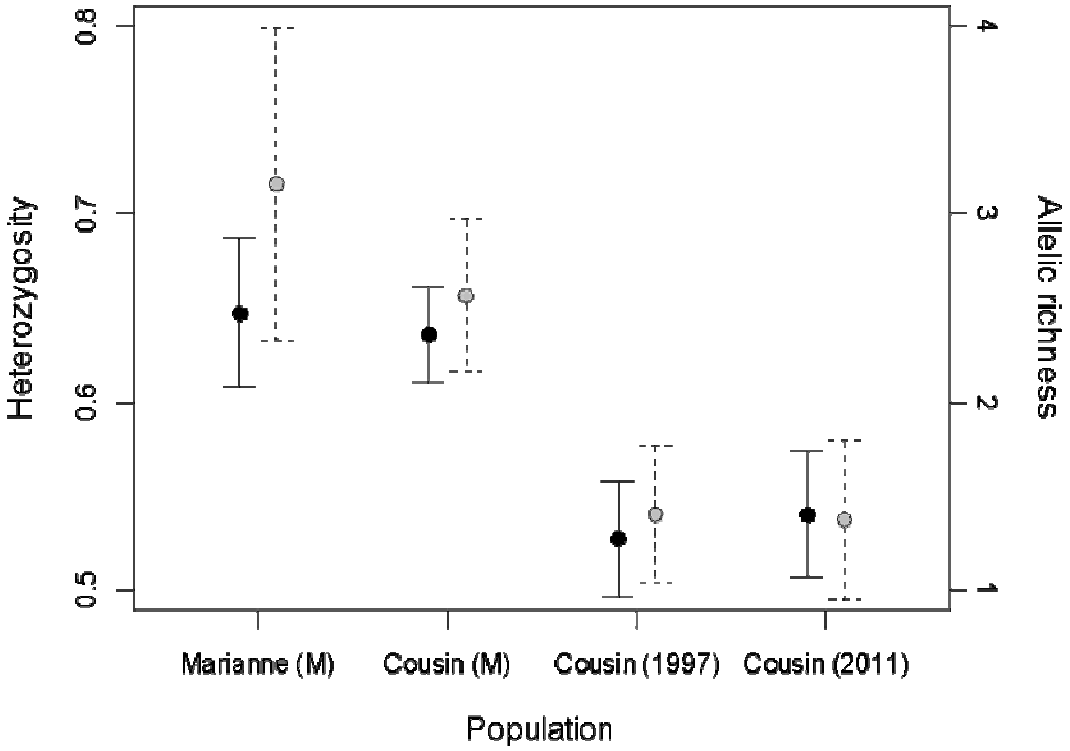

Figure 2.4 Expected heterozygosity (black dots and solid bars) and allelic richness (grey dots and dashed bars) averaged over 12 microsatellite loci in museum (M) and contemporary Seychelles warbler populations. Error bars represent standard error.

Simulations carried out in DIY-ABC strongly supported a bottleneck over the scenario of a constant N_e over time (posterior probabilities = 1 and 0, respectively for both direct and logistic regression estimates). We evaluated confidence in this scenario choice by simulating 500 pseudo-observed datasets under the constant N_e scenario, performing ABC analyses and calculating the number of times the bottleneck scenario was incorrectly chosen, and found that in no case did this occur (suggesting a Type 1 error rate of <0.002). That 14 of the 15

observed summary statistics were well within the range of the simulated data for the bottleneck scenario (Supplementary Table S2.5) gives us further confidence in our scenario choice. Posterior estimates suggested that the Cousin population was reduced from an N_e of several thousand to less than 50 between 120 and 250 years ago, with relatively low bias and high precision estimates (Table 2.1; assuming a generation time of four years). The estimate of ancestral N_e did have wide-ranging credible intervals – a result that could arise from limited signal in the microsatellite data, low sample size in the museum population or a bottleneck occurring before the museum samples were taken. Estimates of N_e in ONeSAMP, like DIY-ABC, gave a much higher value for the museum population than the contemporary population, although absolute estimates were consistently lower (museum population: mean = 268, 95% credible intervals = 175–1320; contemporary population: mean = 32; 95% credible intervals = 27–41). We note, sample sizes lower than 20 individuals can cause problems with ONeSAMP (D. Tallmon, Pers. Comm.), and therefore interpret absolute values from this program with caution.

2.5 Discussion

The Seychelles warbler, like many bottlenecked species, is characterised by low levels of genetic diversity at neutral and functional genes (Richardson *et al.* 2000; Richardson & Westerdahl 2003; Hansson & Richardson 2005); however, until now we have not had confirmation of the timing, extent or duration of this bottleneck. Using museum samples, we found that prior to the 19th Century there was considerably more genetic diversity within and across the only two known ancestral Seychelles warbler populations. Moreover, we show that a substantial proportion of this genetic diversity has been lost over the subsequent decades. Simulations informed with the museum and contemporary population data indicate that the effective population size of the Seychelles warbler has historically been orders of magnitude greater than it is now.

The two main sources of genotyping error that can affect DNA-based studies of museum specimens are allelic dropout and false alleles (Taberlet *et al.* 1996; Wandeler *et al.* 2007). Our modest genotyping error rate of 2.5% in the museum specimens suggests that, while genotyping error has most likely occurred in our museum specimens, it cannot be the explanation for the striking differences in genetic diversity and allelic composition between the historical and contemporary populations (Figure 2.4). Moreover, all available evidence suggests that the allelic drop-out rate overwhelms the rate of false alleles in degraded samples (Wandeler *et al.* 2007; Arandjelovic *et al.* 2009), which in our case would cause an

underestimation of the true pre-bottleneck diversity. With this evidence in mind, combined with the fact that we only included new alleles that were confirmed in replicated PCRs, we believe that our results can be interpreted as providing a minimum estimate of the loss of genetic diversity that has occurred in the Seychelles warbler as a result of the bottleneck.

There are very good records of the population size and status of the Cousin Seychelles warbler population from the 1960s onwards, but very little is known about the range and numbers of the species before then. The Seychelles are thought to have comprised a single, large island when sea levels were lower during the last ice age (Colonna *et al.* 1996; Rocha *et al.* 2013), and we therefore presume that the Seychelles warbler was widely distributed at that time. Our findings suggest that the warbler was indeed widely distributed until relatively recently, with the genetic consequences of the population decline occurring within the last century. The island of Cousin can support a maximum of *ca.* 350 birds (including juveniles); so the large ancestral effective population size estimated here must indicate that warblers were present on neighbouring islands, with gene flow occurring between populations. This at first seems counter-intuitive, as inter-island dispersal is now rarely observed in the Seychelles warbler (Komdeur *et al.* 2004). However, in the past, when many more warblers were present across the Seychelles, rare dispersal events would easily have maintained genetic diversity across small island populations. The finding of moderate differentiation between the Marianne and Cousin museum populations (F_{ST} = 0.13) – two of the most geographically separated islands within the Praslin group (Figure 2.1) – is consistent with a scenario of limited, but not ubiquitous, historical dispersal between islands.

The loss of genetic diversity between museum and contemporary samples, the results from bottleneck tests, and the estimate of the bottleneck time from DIY-ABC all indicate a recent dramatic decline in population size in the Seychelles warbler. Most islands in the Seychelles were planted with coconuts in the late 19th and early 20th centuries, creating unsuitable habitat for Seychelles warblers (Komdeur 1994). This, along with the introduction of predatory rats and cats, drove declines across Seychelles warbler populations. Many other island fauna share a similar history to the Seychelles warbler, i.e. of being extremely rare when discovered in the 19th-20th century, but with little or no information on prior population history such as other Seychelles species, the magpie robin (*Copsychus sechellarum*) and Seychelles paradise flycatchers (*Terpsiphone corvina*; Collar & Stuart 1985). It is likely that many of these species have been subject to the same pressures, and were also recently more widely distributed and abundant (Bristol *et al.* 2013).

Long-term study systems such as the Seychelles warbler provide unique insight into key processes in ecology and evolutionary biology (Clutton-Brock & Sheldon 2010). Now having a historical backdrop against which questions can be addressed allows us to make clearer interpretations of results. For example, there is no evidence that female Seychelles warblers choose either social or extra-pair males to avoid inbreeding (Richardson *et al.* 2004). One potential explanation for this is that inbreeding in this species has purged deleterious mutations, reducing selection for inbreeding avoidance. However, it now seems unlikely that inbreeding in the Seychelles warbler has been severe or long-lasting enough to have purged all deleterious mutations. Alternatively, inbreeding avoidance mechanisms may not evolve in larger populations where inbreeding is infrequent (Jamieson 2011); a more likely scenario given the large ancestral N_e documented here. Likewise, it has been suggested that in birds, bottlenecked island populations exhibit lower levels of extra-pair paternity (EPP) than outbred, mainland species (Griffith 2000). Yet the Seychelles warbler does not fit this pattern, exhibiting higher levels of EPP (40% of offspring) than many outbred species (Richardson *et al.* 2001). However if there is a genetic basis to promiscuity, and the warbler populations were large until recently, we do not necessarily expect low levels of EPP to have evolved.

The approaches used here, combining museum DNA with population simulations, have been and will be of use to other studies wishing to generate clear, testable hypotheses about ecological and evolutionary phenomena. These may be questions similar to those that we address using the Seychelles warbler system concerning inbreeding depression and the evolution of promiscuity. Alternatively they may be questions related to older ecological and evolutionary phenomena, such as the role of climate fluctuations and prehistoric hunting pressures in historical population change (Fontaine *et al.* 2012), or the role of drift, mutation and selection in shaping patterns of genetic diversity (Yeung *et al.* 2011; Spurgin *et al.* 2014). Further, as more studies gain an understanding of population history the generality of ecological and evolutionary hypotheses can be tested using comparative approaches. For example, the relationship between genetic diversity and EPP in birds is at present restricted to analysis of a few, outbred species (Spurgin 2013). Comparing pre and post bottleneck genetic diversity to EPP across a range of species would be a very promising approach to this question, as by doing so one could explore how responsive promiscuity is to sudden changes in population size.

Genetic diversity is crucial for the long-term persistence of populations and species, and is one of the IUCN's three global conservation priorities along with species and ecosystem diversity

(McNeely *et al.* 1990). The rapid loss of genetic diversity, such as that observed here, is therefore a cause for concern, as the evolutionary potential of this species has almost certainly been reduced. Species such as the Seychelles warbler, which have undergone the most rapid and severe reductions in population size, are likely to be at higher risk of extinction in the medium to long term. This is particularly concerning as inbreeding and low genetic diversity has been shown to reduce fitness in the Seychelles warbler (Richardson *et al.* 2004; Brouwer *et al.* 2007; Brouwer *et al.* 2010). Some of the risks associated with low genetic diversity in this species will have been mitigated by the translocations. However, given that N_e was already dramatically reduced when the translocations were carried out, and the further bottlenecks associated with moving a subset of individuals (Chapter 3), it is likely that genetic diversity is still being lost across the Seychelles warbler populations. With this in mind, and given that we now know that there was historical dispersal between warbler populations, there is a strong argument for carrying out assisted gene flow to preserve genetic diversity of this species. As more studies use museum specimens in a conservation context, we will be able to make better-informed conservation decisions. Most importantly, by identifying past changes in demography, it will be possible to identify those populations and species in greatest need of conservation action, and to make evidence-based decisions about what action is most appropriate and how monitoring should be undertaken.

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Supplementary Table S2.1 Collection details of 26 museum specimens of Seychelles warbler and the number of microsatellite loci each sample was genotyped at. The sample highlighted in bold and underlined was excluded from microsatellite analyses.

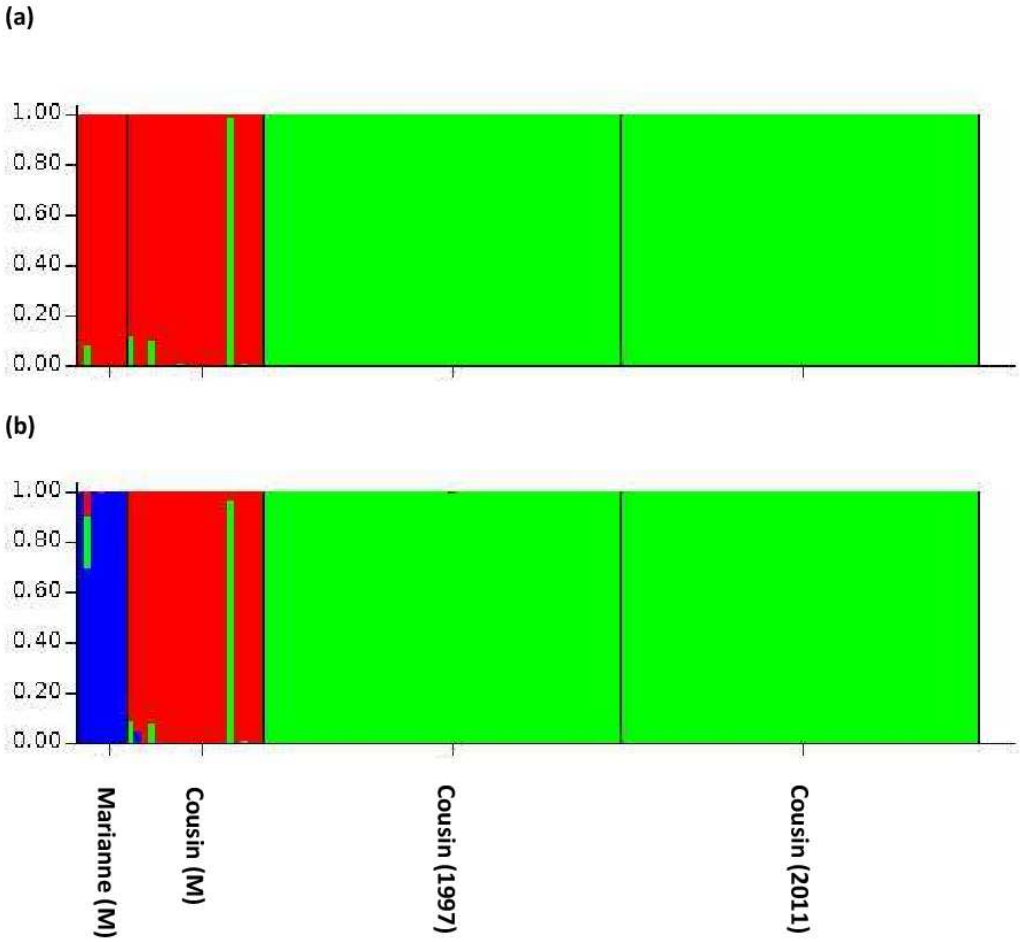
Museum No.	Sex	Collection date	Location	Museum (Location)	Collector	*N*(loci)
119752	M	7 May 1890	Cousin	USNM (Smithsonian)	Abbott	7
119753	F	7 May 1890	Cousin	USNM (Smithsonian)	Abbott	6
265502	M	24 Aug 1904	Cousin	AMNH (New York)	Thaibault	11
596991	M	9 Jul 1904	Cousin	AMNH (New York)	Thaibault	12
596992	M	24 Aug 1904	Cousin	AMNH (New York)	Thaibault	12
596993	M	24 Aug 1904	Cousin	AMNH (New York)	Thaibault	12
596994	M	27 Aug 1904	Cousin	AMNH (New York)	Thaibault	11
596995	F	24 Aug 1904	Cousin	AMNH (New York)	Thaibault	10
596996	F	9 Jul 1904	Cousin	AMNH (New York)	Thaibault	12
1876-377	-	1876	Marianne	MNHN (Paris)	De L'Isle	12
1876-574	**-**	**1876**	**Marianne**	**MNHN (Paris)**	**De L'Isle**	**3**
1878-552	M	1877	Marianne	MNHN (Paris)	Lantz	12
1878-553	M	1877	Marianne	MNHN (Paris)	Lantz	12
CG1938-897	M	1905	Cousin	MNHN (Paris)	Didier	12
CG1938-898	F	1905	Cousin	MNHN (Paris)	Didier	11
CG1938-899	F	1905	Cousin	MNHN (Paris)	Didier	12
1878.7.30.3	M	1878	Marianne	NHM (Tring)	Lantz	11
1927.12.18.391	F	10 Feb 1888	Cousin	NHM (Tring)	Lister	11
1927.12.18.395	F	10 Feb 1888	Cousin	NHM (Tring)	Lister	12
1946.75.23	M	April 1940	Cousin	NHM (Tring)	Sapsworth & Goodfellow	12
1946.75.24	M	April 1940	Cousin	NHM (Tring)	Sapsworth & Goodfellow	12
1946.75.25	F	April 1940	Cousin	NHM (Tring)	Sapsworth & Goodfellow	12
27/Syl/11/b/1	M	September 1877	Marianne	UMZC (Cambridge, UK)	Lantz	12
27/Syl/11/b/2	-	18 February 1888	Cousin	UMZC (Cambridge, UK)	Lister	12
27/Syl/11/b/3	F	September 1877	Marianne	UMZC (Cambridge, UK)	Lantz	11
140287	M	24 Aug 1904	Cousin	MCZ (Harvard University)	Thaibault	10

Supplementary Table S2.2 Primer and multiplex details for all loci tested in museum samples (T_a = annealing temperature).

Primer set	Fluoro-label	Allele size range (bp)	Reference
Multiplex 1 *(T_a = 55ºC)*			
Ase9	NED	130 – 137	Richardson *et al.* (2000)
Ase10	FAM	122 – 143	Richardson *et al.* (2000)
Ase27	NED	184 – 230	Richardson *et al.* (2000)
Ase37	FAM	237 – 247	Richardson *et al.* (2000)
Ase42	NED	249 – 253	Richardson *et al.* (2000)
Ase48	FAM	272 – 284	Richardson *et al.* (2000)
Ase58	HEX	283 – 316	Richardson *et al.* (2000)
Multiplex 2 *(T_a = 55ºC)*			
Ase4	FAM	106 – 108	Richardson *et al.* (2000)
Ase6	FAM	117 – 129	Richardson *et al.* (2000)
Ase13	HEX	140 – 152	Richardson *et al.* (2000)
Ase18	HEX	184 – 196	Richardson *et al.* (2000)
Ase25	FAM	173 – 217	Richardson *et al.* (2000)
Ase35	NED	230 – 234	Richardson *et al.* (2000)
Ase56	FAM	299 – 305	Richardson *et al.* (2000)
Multiplex 3 *(T_a = 56ºC)*			
Ase7	FAM	118 – 122	Richardson *et al.* (2000)
Ase19	FAM	171 – 177	Richardson *et al.* (2000)
Ase24	NED	179 – 183	Richardson *et al.* (2000)
Cuμ4-Gga5	HEX	234 – 238	Hinten *et al.* (unpublished)
PmaTGAn42	FAM	256 – 264	Saladin *et al.* (2003)
Ase55-CEST	HEX	278 – 280	Dawson *et al.* (unpublished)
Ase61	HEX	267 – 390	Richardson *et al.* (2000)
Ase64	HEX	405 – 413	Richardson *et al.* (2000)
Multiplex 4 *(T_a = 57.5ºC)*			
Ase3	HEX	94 – 100	Richardson *et al.* (2000)
Ase11	NED	115 – 127	Richardson *et al.* (2000)
Ase16	HEX	145 – 184	Richardson *et al.* (2000)
Pdoμ6	FAM	205 – 212	Griffith *et al.* (1999)
Ase38	NED	224 – 228	Richardson *et al.* (2000)
Pte24-CEST	HEX	239 – 241	Dawson *et al.* (unpublished)
Ase53	FAM	266 – 287	Richardson *et al.* (2000)
Calex-08-Gga	HEX	344 – 348	Küpper *et al.* (unpublished)

Supplementary Table S2.3 Nucleotide sequences for each previously unpublished primer tested in the museum samples. References: 1 Hinten *et al.* (unpublished data), 2 Dawson *et al.* (unpublished data), 3 Küpper *et al.* (unpublished data)

Primer set	Ref.	Forward sequence (5' – 3')	Reverse sequence (5' – 3')
Cuμ4-Gga5	1	CRKGCAAGMACAAAGCAAAATCC	TTCCTAAAYCTCARRTKGACTCAAG
Ase55-CEST	2	AGCTGGATTGGCATCGTG	TCATTACAGCAATTACCATTGAGC
Pte24-CEST	2	AACAAAGGACGCCGAGTAG	TCATTTAATGGCTYTACTTCATACAT
Calex-08-Gga	3	TTAMAGAATTCTTTCACATGGTCTCT	GTTTCTTCGGAATATTAAGTAGAGGCTTCCAT



Supplementary Figure S2.1 Results from STRUCTURE analyses on 126 Seychelles warbler individuals from museum (M) and contemporary populations. Each vertical line represents an individual, and the colours represent the proportion of each cluster assigned to that individual. Analyses were carried out using **(a)** *K* = 2, and **(b)** *K* = 3 clusters.

Supplementary Table S2.4 Levels of genetic diversity, results of tests for Hardy-Weinberg equilibrium (HWE) and estimates of null allele frequencies at 12 microsatellite loci in museum (M) and contemporary Seychelles warbler populations. *k* = number of alleles, and 'ND' = 'not done' (due to the low sample size on Marianne).

Population	Locus	*k*	$H_{(OBS)}$	$H_{(EXP)}$	$P_{(HWE)}$	$F_{(NULL\ ALLELES)}$
Marianne (M)	Ase10	3	0.57	0.56	0.51	ND
N = 6	Ase58	5	0.50	0.82	0.20	ND
	Ase9	5	0.67	0.80	0.78	ND
	Ase13	3	0.83	0.62	0.64	ND
	Ase25	3	0.50	0.59	1.00	ND
	Ase4	2	0.33	0.49	1.00	ND
	Ase6	5	0.67	0.85	0.43	ND
	Ase61	7	0.67	0.89	0.65	ND
	Ase7	3	0.67	0.62	1.00	ND
	PmaTGA42	3	0.50	0.44	1.00	ND
	Ase11	2	0.33	0.55	0.48	ND
	Ase3	2	0.50	0.53	1.00	ND
Cousin (M)	Ase10	4	0.53	0.70	0.44	0.13
N = 19	Ase58	5	0.71	0.72	0.67	0.01
	Ase9	5	0.59	0.70	0.27	0.08
	Ase13	3	0.47	0.68	0.17	0.16
	Ase25	6	0.74	0.74	0.35	-0.02
	Ase4	3	0.53	0.55	0.07	-0.02
	Ase6	4	0.67	0.69	0.63	-0.01
	Ase61	5	0.57	0.71	0.10	0.10
	Ase7	3	0.53	0.54	0.04	-0.02
	PmaTGA42	2	0.54	0.47	1.00	-0.09
	Ase11	3	0.44	0.62	0.15	0.16
	Ase3	4	0.47	0.51	0.75	0.00
Cousin (1997)	Ase10	3	0.44	0.40	0.12	-0.03
N = 50	Ase58	5	0.62	0.67	0.20	0.03
	Ase9	3	0.38	0.44	0.42	0.06
	Ase13	3	0.54	0.50	0.90	-0.04
	Ase25	5	0.78	0.74	0.91	-0.03
	Ase4	2	0.36	0.39	0.71	0.03
	Ase6	4	0.74	0.67	0.62	-0.05
	Ase61	3	0.44	0.53	0.41	0.09
	Ase7	2	0.66	0.49	0.02	-0.15
	PmaTGA42	2	0.54	0.46	0.35	-0.09
	Ase11	3	0.56	0.54	0.80	-0.02
	Ase3	3	0.56	0.50	0.74	-0.07
Cousin (2011)	Ase10	3	0.40	0.38	1.00	-0.04
N = 50	Ase58	5	0.76	0.72	0.39	-0.03
	Ase9	3	0.44	0.43	0.71	-0.03
	Ase13	3	0.46	0.44	1.00	-0.03
	Ase25	5	0.70	0.72	0.79	0.01
	Ase4	2	0.62	0.48	0.07	-0.13
	Ase6	4	0.72	0.73	0.90	0.00
	Ase61	3	0.52	0.59	0.28	0.07
	Ase7	2	0.52	0.50	1.00	-0.02
	PmaTGA42	2	0.48	0.50	0.78	0.02
	Ase11	3	0.48	0.52	0.69	0.03
	Ase3	3	0.36	0.47	0.16	0.12

Supplementary Table S2.5 Model checking for DIY-ABC analyses. We simulated 10,000 datasets, and for each summary statistic and population calculated the proportion of datasets in which the summary statistic was less than the observed value. Values < 0.05 or > 0.95 indicate a poor match for that particular summary statistic and population, and are highlighted in bold.

Summary statistic	Estimate	*P* (Simulated < Observed)	
		Bottleneck	Constant
Heterozygosity (M)	0.67	0.73	0.91
Heterozygosity (1997)	0.53	0.59	0.08
Heterozygosity (2011)	0.54	0.57	**1.00**
Allelic richness (M)	4.54	0.44	0.63
Allelic richness (1997)	3.15	0.37	**0.01**
Allelic richness (2011)	3.15	0.66	**0.01**
G-W (M)	0.81	0.43	0.56
G-W (1997)	0.66	0.83	**0.02**
G-W (2011)	0.66	0.18	**0.001**
F_{ST} (M-1997)	0.15	0.45	**0.95**
F_{ST} (M-2011)	0.14	0.16	0.92
F_{ST} (1997-2011)	0.01	**0.98**	0.47

Chapter 3

The impact of translocations on neutral and functional genetic diversity within and among populations of the Seychelles warbler

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Frégate Island – about to receive the founders of the fourth translocation

3.1 Abstract

Translocations are an increasingly common tool in conservation. The maintenance of genetic diversity through translocation is critical for both the short- and long-term persistence of populations and species. However, the relative spatio-temporal impacts of translocations on neutral and functional genetic diversity, and how this affects genetic structure among the conserved populations overall, has received little investigation. We compared the impact of translocating different numbers of founders on both microsatellite and major histocompatibility complex (MHC) class I diversity over a 23-year period in the Seychelles warbler (*Acrocephalus sechellensis*). We found low and stable microsatellite and MHC diversity in the source population and evidence for only a limited loss of either type of diversity in the four new populations. However, we found evidence of significant, but low to moderate, genetic differentiation between populations, with those populations established with fewer founders clustering separately. Stochastic genetic capture (as opposed to subsequent drift) was the main determinant of translocated population diversity. Furthermore, a strong correlation between microsatellite and MHC differentiation suggested that neutral processes outweighed selection in shaping MHC diversity in the new populations. These data provide important insights into how to optimise the use of translocation as a conservation tool.

3.2 Introduction

The translocation of populations for species conservation and ecosystem restoration is an increasingly common conservation tool (Moritz 1999; Ewen *et al.* 2012; IUCN/SSC 2013). However, translocation success rates have been poor across many taxa, often for unknown reasons (Griffith *et al.* 1989; Wolf *et al.* 1996; Godefroid *et al.* 2011). Close monitoring of translocated populations is crucial if we are to understand the drivers of success and failure (Miller *et al.* 1999; Allendorf & Luikart 2007), but until recently such monitoring has often been absent or inadequate (Fischer & Lindenmayer 2000; Armstrong & Seddon 2008). Translocations have been particularly widely used in the conservation of oceanic island species. Such islands possess some of the most globally threatened and evolutionarily distinct taxa (Kier *et al.* 2009; Lee & Jetz 2011), contributing disproportionally to global biodiversity (Whittaker 1998). Further, the small size of many oceanic islands means that eradication of alien predators and restoration of native biota are achievable goals (Nogales *et al.* 2004; Howald *et al.* 2007), enhancing the prospects of successful translocations.

Many biotic and abiotic factors can influence translocation success, including genetic diversity (Sarrazin & Barbault 1996; Wolf *et al.* 1998; Seddon *et al.* 2007; Groombridge *et al.* 2012). Maintaining genetic diversity is one of the IUCN's three conservation priorities and its role in species extinction risk is now largely accepted (Spielman *et al.* 2004; Frankham 2005; O'Grady *et al.* 2006). However, translocations typically involve small source populations and limited numbers of founders, resulting in founder effects (e.g. Taylor & Jamieson 2008; Cardoso *et al.* 2009). Furthermore, genetic drift is stronger in smaller populations and will erode genetic diversity (Kimura 1983) and cause inter-population divergence if populations are isolated (e.g. Brekke *et al.* 2011). Small, isolated populations also suffer inbreeding (Frankham *et al.* 2002) and inbreeding depression (Charlesworth & Charlesworth 1987). Purging of the mutational load may alleviate this over time (Crnokrak & Barrett 2002), but its effectiveness in wild populations is uncertain (Boakes *et al.* 2007). In the longer term the loss of genetic diversity also reduces a population's ability to adapt to future challenges, that is its evolutionary potential (Franklin 1980; Franklin & Frankham 1998). Translocations can, therefore, have considerable long-lasting genetic impacts on populations, and hence on entire species (Biebach & Keller 2009) and there is a clear need to integrate genetic considerations into translocation programmes (Jamieson & Lacy 2012).

Most studies on the genetic impacts of translocation investigate diversity at putatively neutral markers such as microsatellites (e.g. Larson *et al.* 2002; Taylor & Jamieson 2008; Brekke *et al.* 2011). Such variation may not correlate well with the genetic diversity important to the

evolutionary potential of the new populations (Reed & Frankham 2001; Aguilar *et al.* 2004). It may be more relevant to directly assess variation at important functional loci, such as the major histocompatibility complex (MHC). These highly polymorphic loci encode molecules central to antigen recognition in the adaptive immune response of vertebrates (Hughes & Yeager 1998). This key role has made the MHC an attractive candidate for studying functional diversity in and among populations (reviewed in Bernatchez & Landry 2003; Sommer 2005; Spurgin & Richardson 2010). A few studies have compared the impact of translocation on neutral and functional diversity in wild populations (see Sutton *et al.* 2011; Bauer *et al.* 2013; Monzón-Argüello *et al.* 2013). Perhaps the best example to date is a study by Miller and Lambert (2004) which showed that drift outweighed selection in shaping MHC diversity in re-introduced populations of two bird species in New Zealand. However, the reasons for differences in loss of neutral and functional variation during the process of translocation itself remain unclear.

How many individuals to translocate is a difficult question to answer, although it is a central concern for conservationists. Guidelines recommend that 'adequate' numbers of founders should be taken (IUCN/SSC 2013), although this is rarely quantified (Tracy *et al.* 2011). The goal of translocation is to establish a 'viable' population (IUCN/SSC 2013), often defined by the 'minimum viable population' concept, with estimates ranging from 50 individuals for short-term persistence up to 5,000 individuals to maintain evolutionary potential (Franklin 1980; Lande 1995; Franklin & Frankham 1998; Willi *et al.* 2006). However, translocations often use <50 founders owing to ecological, logistic and economic constraints (Komdeur 1994; Cardoso *et al.* 2009; Jamieson 2011; Tracy *et al.* 2011). Genetic data have only recently been incorporated into estimates of what constitutes 'adequate' founder sizes, even though the genetic consequences of small population size are directly relevant. Weeks *et al.* (2011) introduced the concept of 'genetic capture', where the minimum number of individuals to translocate is determined by the capture of ≥95% of source population genetic diversity. Models have also been developed to predict allele retention accounting for post-translocation parameters such as survival, population growth, carrying capacity, overlapping generations and/or specific mating systems (Brekke *et al.* 2011; Tracy *et al.* 2011; Weiser *et al.* 2012). Modelling approaches provide vital benchmarks for conservationists but there are drawbacks. Importantly, these methods are largely based on the loss of neutral variation in the founding population. Drift may disproportionately reduce variation at functional loci if they are highly variable due to a history of balancing selection (Sutton *et al.* 2011). Therefore such methods are only useful for functional diversity if the loci are selectively neutral at small population sizes (Tracy *et al.* 2011). Complementing modelling approaches with replicated empirical data

on neutral and functional variation, in systems with adequate pre- and post-translocation sampling, should help inform best practice for future translocation programmes.

Here, we present data from a study spanning 23 years and involving four translocations of different founder sizes of the Seychelles warbler (*Acrocephalus sechellensis*), an isolated island species. The objective was to compare the spatio-temporal impact of translocation on neutral and functional diversity within and across populations. First, we characterise neutral and functional genetic diversity in the source population and how that has changed over time. Second, we compare predicted vs. observed levels of genetic capture across different founder sizes. Third, we quantify genetic diversity in the four newly translocated populations, including how that compares with variation in the source population and if it changes over time. Fourth, we quantify levels of genetic differentiation among all the Seychelles warbler populations now in existence. Fifth, we estimate the effective population size for each population. We discuss the implications of our findings for the use of translocation in the conservation of this and other endangered species.

3.3 Materials and methods

3.3.1 Study populations

By the mid 20th century the Seychelles warbler was on the verge of extinction due to habitat destruction and the introduction of invasive predators, with the last population of 26-50 individuals (Crook 1960; Chapter 2) existing on Cousin Island (4°20'S, 55°40'E, 0.29 km²). This population has been under intense study since 1986 (>96% individuals ringed since 1997, Richardson *et al.* 2001) as part of a long-term project (Komdeur 1992; Richardson *et al.* 2007; Barrett *et al.* 2013). Each year, birds are caught and unringed individuals are identified with a unique combination of coloured leg rings (herein referred to as catch-year samples). A *ca.* 25 µl blood sample is taken from every bird and stored in absolute ethanol. Four translocations have been undertaken as part of the species conservation plan (Richardson 2001), with Cousin as the source for each (Supplementary Figure S3.1). In brief, 29 birds were translocated to both Aride (4°12'S, 55°40'E, 0.68 km²) and Cousine (4°21'S, 55°39'E, 0.25 km²) in 1988 and 1990 respectively (Komdeur 1994). In 2004, 58 birds were translocated to Denis (3°48'S, 55°40'E, 1.42 km², Richardson *et al.* 2006) and in 2011, 59 birds to Frégate (4°35'S, 55°56'E, 2.19 km², Wright & Richardson 2012). For each translocation, founders were selected from across the whole source population based on body condition, age (avoiding very young or old birds), breeding experience and sex, in order to maximise the chances of population establishment. The translocations were undertaken blind in regards to genetic characteristics, although translocating known first-order relatives was avoided. Movement between populations is

virtually absent, with rates of inter-island dispersal at only 0.1% (Komdeur *et al.* 2004). Complete sampling of the founding populations was conducted for Denis and Frégate, but the Aride and Cousine translocations were undertaken prior to routine blood sampling and so few founders were sampled (4 of 29 in each translocation). The following catch-year samples were used in this study: Cousin 1993, 2005, 2011; Aride 1993, 2005, 2011; Cousine 1997, 2005, 2011; Denis 2004, 2011; Frégate; 2011 (sample sizes in Table 3.1).

3.3.2 Molecular and statistical analyses

Statistical analyses were performed using R v.2.15 (R Development Core Team 2012), unless stated. Samples were genotyped at 30 microsatellite loci following chapter 2. Rare alleles (frequency <0.01) were verified by two or more independent PCRs from different samples. We tested for deviations from Hardy-Weinberg equilibrium and linkage disequilibrium between loci using GENEPOP v.4.1 (Raymond & Rousset 1995b) and for null alleles using CERVUS v.3.0 (Marshall *et al.* 1998). Genetic diversity in each catch-year sample on each island was quantified by calculating expected heterozygosity (H_E) using ARLEQUIN v.3.5 (Excoffier & Lischer 2010). Allelic richness was quantified using a rarefaction approach in HP-RARE v.1 (Kalinowski 2005) as sample size differences can bias the estimations (Leberg 2002).

Variation at exon 3 of MHC class I, which codes for the peptide binding region involved in antigen recognition (Hughes & Yeager 1998), was screened using reference-strand mediated conformation analysis (RSCA) with the primers from Richardson & Westerdahl (2003), following the method of Worley *et al*. (2008). Each segregating RSCA variant corresponded to a unique 255 bp amino acid coding sequence (hereafter termed “allele” for simplicity, Richardson & Westerdahl 2003). Ten MHC class I alleles have been detected in the Seychelles warbler, with individuals possessing 2–8 alleles each, suggesting that at least four class I loci are amplified (Richardson & Westerdahl 2003). Our primers were sited within exon 3. Consequently, we were not able to screen all the variation within this exon and it is possible that some additional polymorphism exists (e.g. Llaurens *et al.* 2012). However, the amplicon includes all the codons of the peptide-binding region where we expect most variation to be found (Hughes & Yeager 1998). Further, to minimise the effect of this issue we employed two primer sets which vary at the 3’ end where a known polymorphism occurs (see Richardson & Westerdahl 2003). Finally, any missed variation would not affect the main results or conclusions of the present study, which seeks to address how the variation we have screened is captured across translocated populations. It is impossible at present to identify locus zygosity, due to homogeneity of alleles between multiple, duplicated loci within the MHC (Westerdahl 2007). Instead, we measured MHC diversity by calculating the total number of

different alleles in each catch-year sample, the mean number of alleles per individual (MHC/ind), nucleotide diversity (Pi) and theta *K* (allelic richness) in ARLEQUIN by entering the nucleotide sequence and number of individuals carrying each allele as haplotype data, following Miller *et al.* (2010). This approach may overestimate rare alleles and underestimate common ones but is the best available (Ekblom *et al.* 2007).

Changes in genetic diversity over time were analysed using a randomisation approach (Manly 1997). For each diversity measure the data from the earliest and latest years of each population were pooled and randomly re-sampled with replacement 100,000 times. The *P* value was calculated as the proportion of times the difference between the means of the re-sampled datasets were equal to or greater than the observed difference between earliest and latest years. Differences in microsatellite variation between years were also tested using a global differentiation exact test (Raymond & Rousset 1995a) in ARLEQUIN, with a 30,000 step Markov chain. Seychelles warblers can live up to 17 years (Brouwer *et al.* 2010) and are routinely sampled multiple times throughout their life as part of this long-term study system (e.g. Barrett *et al.* 2013). To check for any effect of including the same individuals across catch-year samples, diversity measures were also compared for lay-year (year of hatching, estimated at first ringing) cohorts on Cousin. Patterns of diversity across lay-years and catch-years were qualitatively the same (data not shown) so only catch-year data are reported. This enabled us to use the largest sample sizes available and hence most accurate estimations of diversity for each year in our analyses. Differences in diversity between populations were assessed using the same randomisation approach. As we observed no differences in variation within islands over time (see Results), for between-island comparisons samples were pooled for each island to improve the accuracy of the rarefaction measures (i.e. allelic richness). Differences in mean MHC/ind between islands were tested using a Kruskal-Wallis test.

To model the expected genetic capture at each translocation, we constructed rarefaction curves of observed allelic richness using the 2011 Cousin sample (microsatellites; n = 163, MHC; n = 91) with 1,000 repetitions. As we did not have source population diversity data prior to every translocation and diversity did not change over time within islands (see Results), we used the 2011 sample as a proxy for pre-translocation diversity at all four genetic capture events. A proportionally greater loss of genetic diversity is expected from microsatellite loci of higher initial diversity (Hedrick 1999), so microsatellite genetic capture was investigated using two models: all loci (n = 30) and 'diverse loci' only (n = 7), defined as possessing ≥4 alleles, a threshold that separates out the *ca.* 25% most polymorphic loci in our dataset. As mean MHC/ind varied over time (see Results) a second MHC curve was constructed using the 1993

Cousin sample (n = 52) to check the accuracy of capture estimates for the earlier translocations to Aride (1988) and Cousine (1990). The rarefaction curves were then used to estimate the expected allelic richness captured during the translocations by taking the mean of the 1,000 rarefaction repetitions for each number of translocated birds (Aride/Cousine 29, Denis 58, Frégate 59). To test model accuracy, we compared the observed microsatellite allelic richness captured for Denis and Frégate (where we had complete founder samples) with the distribution of expected values generated by rarefaction. A one-tailed *P* value was obtained by calculating the percentile of simulated data in which the observed mean was located.

Population structure within and among islands was tested by calculating microsatellite and MHC pair-wise F_{ST} in ARLEQUIN across all twelve catch-year samples. We also calculated pair-wise D_{EST} (Jost 2008), using the R package MMOD (Winter 2012) for microsatellites and SPADE (Chao & Shen 2010) for MHC. The measures were strongly correlated for both microsatellites and MHC (both $r_M > 0.90$, $P < 0.001$) hence only F_{ST} is reported. The relationship between pair-wise microsatellite and MHC F_{ST} values was assessed using a Mantel test. A Bayesian algorithm was also implemented in STRUCTURE v.2.3 (Pritchard *et al.* 2000) to determine the most likely number of genetic clusters (K). As all islands have recent common ancestry, limited differentiation was expected, so samples from the latest sampling period (2011) were used along with a model of no admixture, prior information on sampling location and correlated allele frequencies, features which are suited to detecting subtle structure (Hubisz *et al.* 2009). We carried out four independent iterations of 500,000 repetitions with a burn-in of 20,000 at each clustering level for K = 1–5. We analysed the results using STRUCTURE HARVESTER (Earl & vonHoldt 2012), which implements the *ad-hoc* ΔK test (Evanno *et al.* 2005), a more accurate estimate of the most likely number of clusters than assessing log probability alone. The STRUCTURE results were visualised using DISTRUCT v. 1.1 (Rosenberg 2004).

Estimates of the effective population size (N_e) of each population were obtained using two different methods. (1) We used the approximate Bayesian computation approach implemented in ONeSAMP (Tallmon *et al.* 2008), with N_e priors of 2-150 for each island. This method bases N_e on a single population sample (from 2011). (2) We used a linkage disequilibrium approach in LDNE (Waples & Do 2008) with a random mating model to account for complex patterns of breeding in this species (Komdeur 1992; Richardson *et al.* 2001). The lowest allele frequency allowed was 0.02 to address the trade-off between estimate precision and bias (Waples & Do 2008), although bias should be minimal given the comparatively low polymorphism observed in the microsatellites.

3.4 Results

Microsatellite genotypes were compiled for 658 individuals and MHC genotypes for 581 individuals across the five populations. All populations were in Hardy-Weinberg equilibrium at all microsatellite loci. Linkage disequilibrium was detected in nine different loci pairs across the five islands after sequential Bonferroni correction (26/4,933 pair-wise comparisons significant). However, inconsistency of patterns between and within populations suggests they are not truly linked. Null allele frequency estimates were also inconsistent across years within each island, with no locus frequency (F) ≥0.1 more than twice except for locus *Ase3* (F ≥0.1 in four catch-year samples, Supplementary Table S3.1). Excluding the *Ase3* locus did not qualitatively alter the results (data not shown). All 30 loci were retained in the final analyses. Microsatellite diversity in the source population on Cousin had an overall mean (± SE) H_E of 0.49 ± 0.005 and allelic richness of 2.92 ± 0.01. Neither measure differed across years (randomisation tests, H_E: P = 0.67; allelic richness: P = 0.85) and the global exact test found no overall differentiation across years (P >0.99). Mean (± SE) MHC/ind on Cousin increased from 1993 (3.93 ± 0.19) to 2011 (4.71 ± 0.16, P = 0.003). Full diversity estimates are given Table 3.1.

Table 3.1 Catch-year sampling regime and marker summary statistics for microsatellite and MHC data across the five Seychelles warbler populations. Cousin is the source of all other populations. Abbreviations are number of individuals scored (N), expected heterozygosity (H_E), total number of MHC alleles in the population (Alleles), mean MHC alleles per individual (MHC/ind), nucleotide diversity (Pi) and an index of allelic richness (Theta K).

Island	Catch Year	Microsatellites			MHC				
		N	H_E	Allelic richness	N	Alleles	MHC/ind	Pi	Theta K
Cousin	1993	49	0.49	2.96	52	10	3.94	19.76	2.05
	2005	169	0.48	2.89	156	10	4.61	19.71	1.54
	2011	163	0.48	2.91	91	10	4.71	19.50	1.71
Aride	1993	27	0.49	2.82	27	10	4.85	18.84	2.34
	2005	30	0.47	2.84	30	10	4.67	18.68	2.30
	2011	29	0.45	2.81	29	10	4.93	18.97	2.28
Cousine	1997	24	0.45	2.60	23	10	5.09	19.70	2.43
	2005	29	0.42	2.59	29	9	4.03	19.13	2.10
	2011	30	0.45	2.70	30	10	4.17	20.16	2.05
Denis	2004	58	0.50	2.95	56	10	4.77	19.69	1.91
	2011	35	0.48	2.85	30	10	4.97	19.25	2.25
Frégate	2011	59	0.49	2.90	58	10	4.55	19.52	1.92

Resampling from the source population, the genetic capture model estimated that 95% (3.07 ± 0.01) of the overall microsatellite allelic richness would be captured with the translocation of 59 individuals, but only 87% (4.86 ± 0.01) of allelic richness for diverse loci. Virtually identical values were observed for 58 individuals (all loci = 3.06 ± 0.01, diverse loci = 4.86 ± 0.01). There was no difference between expected and observed allelic richness translocated to Frégate (all

loci: *P* = 0.36, diverse loci; *P* = 0.37, Figure 3.1a) or Denis (all loci: *P* = 0.35, diverse loci: *P* = 0.35), indicating a good fit of the genetic capture models. It is therefore reasonable to assume that the rarefaction curve estimates of genetic capture for 29 individuals (all loci = 91%, 2.94 ± 0.10; diverse loci = 85%, 4.45 ± 0.10) provide an accurate representation of genetic diversity initially captured from the source population during the translocations to Cousine and Aride. Across all 30 loci we observed a loss of eight and ten alleles in the Denis and Frégate translocations respectively. On Denis all losses were of rare alleles (frequency <0.01 in source population) from diverse loci, on Frégate two alleles were also lost from less diverse loci, including the bi-allelic *Pte24*-CEST locus, leading to fixation of a single allele in this population. The lack of founder genotypes or complete population sampling for Aride and Cousine means we could not directly determine the exact roles of genetic capture versus subsequent drift in allele loss for these two translocations.

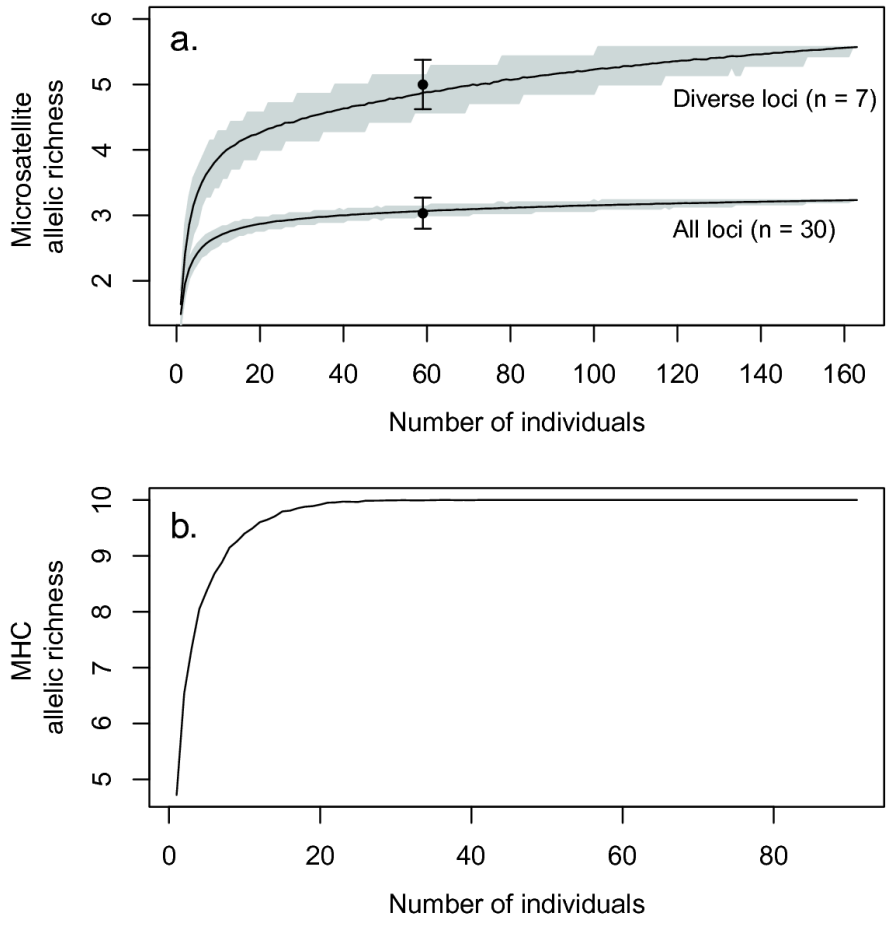

Figure 3.1 Rarefaction curves constructed from 1000 replicates of the genetic variation observed on Cousin Island in 2011. **A)** Microsatellite allelic richness (n=163). Diverse loci have ≥4 alleles and the shaded areas lie between the 5-95th percentiles. Observed mean allelic richness of Frégate founders (n = 59) given as points with SE bars, **B)** MHC variation (n = 91).

The MHC rarefaction curves estimated that *ca.* 20 (2011 sample) to *ca.* 25 (1993 sample) individuals would be required for complete sampling of known class I variation (Figure 3.1b). All MHC class I alleles were subsequently found in the Denis and Frégate founders at translocation and in Aride and Cousine in subsequent catch-year samples (Table 3.1). Diversity estimates for translocated populations are given in Table 3.1. Although some microsatellite alleles were lost in the translocation process, no significant differences in either microsatellite H_E or allelic richness across either the full suite of loci or diverse loci alone were detected between islands (pooled samples, all P >0.20, Figure 3.2). There were also no differences in microsatellite diversity over time within each population (all P >0.30). There was no difference in mean MHC/ind (H = 6.03, P = 0.20) between islands (pooled samples). Similarly there were no differences in MHC/ind across time within each translocated population (all P >0.07).

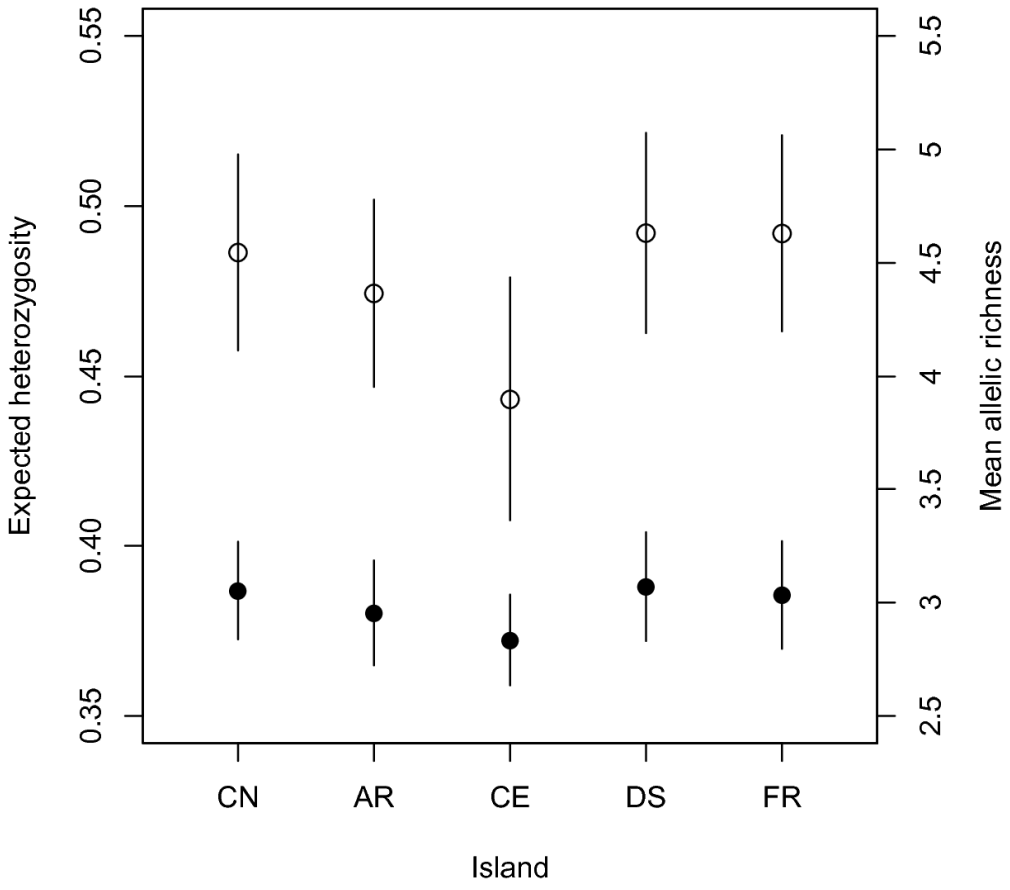

Figure 3.2 Genetic diversity and differentiation between populations of the Seychelles warbler; H_E (white points) and rarefied allelic richness (black points) across all microsatellite loci. Error bars given are SE. CN=Cousin, AR=Aride, CE=Cousine, DS=Denis, FR=Frégate.

Microsatellite pair-wise F_{ST} analyses revealed moderate differentiation between the populations on Aride and Cousine (2011, F_{ST} = 0.08, P <0.001). Subtle differentiation was also detected between all other pair-wise comparisons (2011, F_{ST} = 0.01–0.05, all P <0.001) except

for between Cousin and Frégate (*P* = 0.73). The MHC F_{ST} analyses also revealed subtle differentiation between Cousine and Aride (2011, F_{ST} = 0.02, *P* = 0.002), Denis (2011, F_{ST} = 0.02, *P* = 0.005) and Cousin (2011, F_{ST} = 0.01, *P* = 0.03). Pair-wise F_{ST} are given in Supplementary Table S3.2. A positive correlation was found between microsatellite and MHC F_{ST} (r_M = 0.69, *P* <0.001, Figure 3.3). The STRUCTURE analysis identified two genetically distinct clusters across the five populations (Supplementary Figure S3.2). When visualised, the islands of Cousin, Denis and Frégate contained a mixture of both clusters, but clear segregation of the clusters was observed in the Aride and Cousine populations (Figure 3.4). Estimates of N_e varied between methods (Table 3.2) but both methods estimated Cousin to have the largest N_e and Cousine to have the smallest N_e.

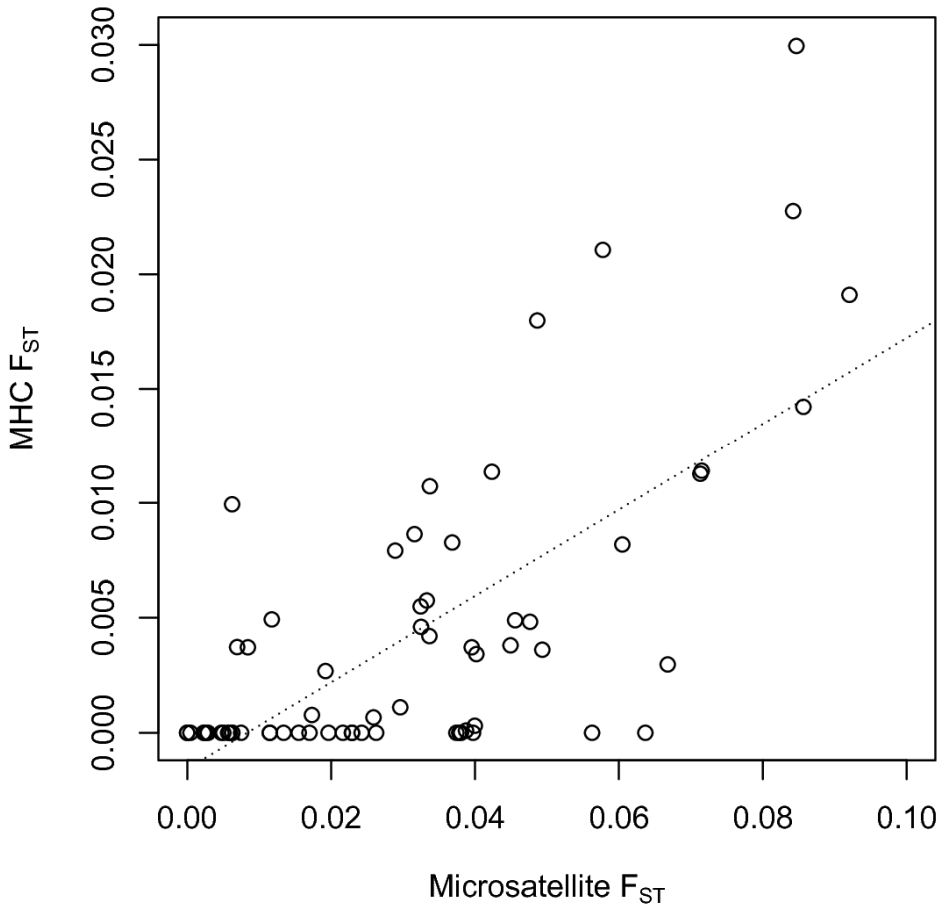

Figure 3.3 Correlation of microsatellite and MHC F_{ST} differentiation across catch-year samples between populations of the Seychelles warbler. Mantel test r = 0.69, *P* <0.001 (using all island and catch-year pair-wise comparisons).

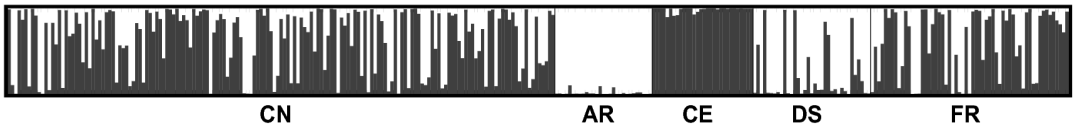

Figure 3.4 STRUCTURE plot of genetic clustering across the five Seychelles warbler populations in 2011; CN, n = 163. AR, n = 29. CE, n = 30. DS, n = 35. FR, n = 59. Two clusters represented by grey and white bars.

3.5 Discussion

The Seychelles warbler, with its translocation history and long-term sampling regime, presents a useful case study of the spatio-temporal impact of conservation translocations on neutral and functional genetic diversity. We found low and temporally stable microsatellite diversity and low MHC diversity in the source population on Cousin. Our rarefaction models predicted allele retention accurately, and genetic capture (as opposed to subsequent drift) appeared to be the main determinant of translocated population diversity. A small number of rare alleles from neutral markers were lost during translocation, but no statistically significant loss of either neutral or functional diversity was detected in any of the four new populations. Further, diversity in these new populations remained stable over time. Importantly, however, the populations established with a lower number of founders (Aride and Cousine) were central to the low to moderate levels of genetic differentiation observed among populations, indicating that translocations produced subtle changes in allele frequencies, if not in levels of diversity *per se*.

Table 3.2 Effective population size estimates for five Seychelles warbler populations using two methods on samples from 2011. Medians are given with credible limits for ONeSAMP and 95% confidence intervals for LDNE.

Island	ONeSAMP	LDNE
Cousin	35 (31 - 42)	68 (59 - 82)
Aride	24 (21 - 31)	39 (26 - 70)
Cousine	23 (21 - 28)	22 (16 - 34)
Denis	28 (25 - 34)	36 (26 - 61)
Frégate	26 (23 - 30)	54 (38 - 85)

Conservationists have been advised to capture ≥95% of the source population's genetic diversity during translocations to limit any bottleneck effects caused by the translocation process (Weeks *et al.* 2011). Our rarefaction models show that translocations to Denis and Frégate (58 and 59 birds respectively) captured *ca*. 95% of neutral diversity (87% for diverse loci), but the earlier translocations of 29 birds to Aride and Cousine only captured *ca.* 91% (85% for diverse loci). In line with this we found a slight, albeit non-significant, decrease in both allelic richness and heterozygosity in the Aride and Cousine populations (Figure 3.2). We would expect a more pronounced effect of loss on allelic richness measures than heterozygosity (Allendorf 1986) but we see no significant difference. A similar study by Taylor & Jamieson (2008) reported little subsequent loss of genetic variation in serial translocations of saddlebacks (*Philesturnus carunculatus*) involving even smaller numbers of founders (lowest n = 16), although the source population also had lower initial variation. Our result supports their suggestion that long-term population history (i.e. previous severe bottlenecks) may negate any contemporary bottleneck effects caused by small numbers of founders. This is simply because if all but the commonest alleles are already lost, there is little variation left to lose in subsequent bottlenecks. However this will only be true for the most extreme cases of genetically depauperate populations (as in the saddlebacks). The Seychelles warbler is severely bottlenecked, and yet we still find some loss of variation during translocations, indicating that care – and suitably high numbers of founders – must be taken to avoid further genetic impoverishment of translocated populations. Furthermore, statistical and biological significance may not concur, as rare alleles can play import roles in evolution (Allendorf 1986). Indeed, another approach to conserving genetic diversity in translocations is to maximise the probability of retaining rare alleles in the founding population (Weiser *et al.* 2012). This would require much larger founder sizes – in our case, our rarefaction curves suggest that in order to capture 99% of variation (thus capturing the majority of rare alleles) we would have needed to translocate in excess of 130 individuals. However, when planning translocations conservation managers are inevitably faced with balancing genetic factors against other considerations (namely logistics, expense and restricted source populations), and the best genetic approach may not be possible. Where neutral markers are a good proxy for functional diversity (as our study suggests can be the case in bottlenecked populations) the development of predictive models of neutral diversity loss makes it possible to compare the genetic consequences of different management options prior to translocation (Weiser *et al.* 2013) – a practice that we would encourage wherever possible.

Disentangling the impact of genetic capture, its associated founder effects, and subsequent drift on genetic diversity is generally not possible, requiring virtually complete sampling of a

population before, during and, for extended periods, after the translocation event. Complete microsatellite genotypes of all founders on Denis and Frégate enabled us to identify genetic capture as the main determinant of diversity in the translocated populations, and to pinpoint alleles lost specifically through genetic capture. All but two of the alleles that were lost were rare (frequency <0.01) in the source population, in line with theoretical expectations (Allendorf 1986; Stockwell *et al.* 1996). The fixation of an allele at locus *Pte24*-CEST on Frégate further demonstrates the stochastic nature of genetic capture. This locus became fixed in the largest founding population, whilst remaining bi-allelic across three other translocations of smaller founder numbers. Our data demonstrate that the loss of rare alleles in the founding populations is due to incomplete genetic capture. Additionally, there is little evidence for significant changes in diversity over time, suggesting that drift subsequent to the translocations has had little effect on these populations. This may partly be explained by the rapid population growth observed in the translocated populations, which will have limited the effects of drift (Nei *et al.* 1975).

The rarefaction models for MHC diversity suggested that *ca.* 20–25 Seychelles warblers would be required to capture all the known source MHC variation, which was achieved in all translocations. MHC copy-number variation may exist in these populations, but it is difficult to separate from the variance in number of alleles shared across duplicated loci (e.g. Eimes *et al.* 2011). It may therefore be logical to translocate individuals with the largest number of different alleles, irrespective of whether this was the result of copy-number variation or across-loci heterozygosity, as this would maximise the MHC variation in the founding population.

The Seychelles warbler possesses a depauperate MHC diversity compared with other *Acrocephalus* species, with only ten class I alleles observed (Hansson & Richardson 2005) and no class II variation detected (Hutchings 2009). Pathogen-mediated balancing selection is believed to maintain MHC diversity in large populations (Doherty & Zinkernagel 1975; Slade & McCallum 1992; Spurgin & Richardson 2010). However, with decreasing population size the effects of drift are more severe and selection needs to be stronger to maintain diversity (Kimura 1983). Studies have shown that neutral processes outweigh selection in shaping MHC diversity in small, bottlenecked and/or isolated populations (e.g. Seddon & Ellegren 2004; Miller *et al.* 2010; Agudo *et al.* 2011; Sutton *et al.* 2011), although instances of selection acting to maintain MHC diversity have been documented (e.g. Aguilar *et al.* 2004, Oliver & Piertney 2012). Here, we find that the pattern of microsatellite and MHC differentiation is highly positively correlated (Figure 3.3), suggesting that any MHC-based selection that occurs in the

Seychelles warbler (Richardson *et al.* 2005; Brouwer *et al.* 2010) is not strong enough to override the effect of neutral processes during translocations. Instead, our data support the conclusion that demographic processes shape both neutral and functional diversity in a similar way. In our case, it seems that the stochastic process of genetic capture of small founder numbers has been the main driver of differentiation between translocated Seychelles warbler populations (Figure 3.4). Although our analysis clearly suggests k = 2 genetic clusters across the populations, it can be interpreted as three 'groupings': the heterogeneous populations of Cousin, Denis and Frégate forming one group and Aride and Cousine each forming another, with the latter two diverging in opposite directions to one another due to their smaller numbers of founders.

Overall, there appears less variation in the MHC than observed at the microsatellites. Admittedly, these particularly variable microsatellites were originally selected from a larger panel of markers to resolve parentage, which may explain their sensitivity in detecting population differentiation, where MHC was less suited. An obvious but key point is that the observable impact of translocations on genetic diversity is wholly dependent on the variability of the loci used in the study. Careful choice of good, highly polymorphic markers is therefore important to enable higher resolution in studies investigating genetic variation loss.

Defining a 'successful' translocation is difficult and can lead to misinterpretation regarding long-term possibility of failure and, potentially, to inadequate future conservation effort (Griffith *et al.* 1989; Fischer & Lindenmayer 2000). Two biologically relevant aspects on which success can be judged are persistence and resilience (Fischer & Lindenmayer 2000). Many avian translocation studies report high mortality during/immediately following release and often complete failure of populations to become established (e.g. Brekke *et al.* 2011; Jamieson 2011; White *et al.* 2012). The Seychelles warbler programme is therefore unusual, with no mortality occurring during any of the four translocations (Komdeur 1994; Richardson *et al.* 2006; Wright & Richardson 2012). No loss of variation is observed subsequently in the translocated populations (after the loss due to the initial genetic capture). This indicates that survival is high, that reproductive representation of founders is balanced, and that the rapid population growth observed during establishment has limited the severity of the founder bottleneck. Current census population estimates for each island are Cousin = 320, Aride = 1850, Cousine = 210, Denis = 300 and Frégate = 80. The populations on Cousin, Aride and Cousine are at carrying capacity (DSR, pers. obs.), with differences in the area and quality of habitat on the different islands responsible for the variation in island capacity. Denis and Frégate are also still in the early stages of post-translocation population growth. The

translocations can therefore be considered extremely successful in the short-medium term. The long-term genetic resilience of a population will depend on the functional variation present. Other bottlenecked species, such as southern elephant seals (*Mirounga leonina*), Chatham Island black robins (*Petroica traversi*), cheetah (*Acinonyx jubatus*) and falcons (*Falco* spp.) survive despite extremely low MHC variability (Slade 1992; Miller & Lambert 2004; Castro-Prieto *et al.* 2011, Gangoso *et al.* 2012). Decreased pathogen exposure within such populations may partly explain their apparent viability (Slade 1992; Miller & Lambert 2004) and depauperate parasite loads are found in the Seychelles warbler (Hutchings 2009). However, whether the remaining MHC diversity provides adequate resilience against any novel pathogens that may enter these populations in the future is unknown.

The variability in N_e estimates between the methods means caution should be exercised with interpretations. Both the severity and duration of bottleneck events will affect estimates of N_e (Nei *et al.* 1975; Frankham 1995). Although the bottlenecks were relatively severe (29 and 58/59), all founders were sourced from an already bottlenecked population (Chapter 2). Further, each population has experienced rapid growth over many generations (with the exception of Frégate, only established at the end of 2011), which would limit further reduction of N_e. The general scale and order of N_e estimates across populations therefore appears logical in relation to known history, as well as founder and census population sizes. Our results show that while the translocations have clearly been very successful in massively expanding the overall Seychelles warbler population and range without significant extra loss of diversity, most of the estimates of N_e for our populations fall below any recommended minimum viable sizes (50-5000, Lande 1995; Franklin & Frankham 1998). This and the low levels of diversity at both neutral and functional loci means that concerns regarding the evolutionary potential of this species still cannot be discounted. Given these results and the evidence that some genetic divergence exists, assisted gene flow between translocated populations may be required in the future to maintain all the Seychelles warblers as one large, undifferentiated and hence more viable population. The general environmental conditions are similar between islands (indeed host islands were selected based on their similarity to Cousin), but we cannot fully discount the possibility of different selection pressures across populations due to factors we have not been able to assess. It may therefore be logical to check for adaptive differences between populations before undertaking assisted gene flow as this could cause outbreeding depression, an important consideration for translocation projects in general (Edmands 2007).

3.5.1 Conclusion

The demographic history of the Seychelles warbler is typical of many endangered species (Hudson *et al.* 2000; Robertson *et al.* 2009; Grueber & Jamieson 2011; Bristol *et al.* 2013) and our results should therefore be of general applicability. Questions have been raised about inferring functional variation based solely on any one given locus, i.e. the MHC (Acevedo-Whitehouse & Cunningham 2006; Radwan *et al.* 2010). Future work on other important immune genes, such as toll-like receptors (e.g. Grueber *et al.* 2013), individual variation in gene copy number (e.g. Eimes *et al.* 2011) and broader genomic studies (Angeloni *et al.* 2012), will help our understanding of how and why genetic variation influences population persistence. What role candidate genes versus genomic approaches will play in conservation programmes will also undoubtedly be an important avenue of research. However conservation biology is a crisis discipline (Soulé 1985) and requires efficient, evidence-based decision-making. From a practical perspective, in the case of the Seychelles warbler, investigating MHC variation as an example of functional genetic diversity provided no extra information (above and beyond that of the microsatellite data) to help estimate the numbers of founders required. Evidence is now accumulating for drift outweighing selection in small populations and that maintaining maximal genetic diversity is vital in species conservation. Based on this, we suggest that in bottlenecked, isolated species, such as the Seychelles warbler, genetic decisions on the number of founders to use in translocations could be made most efficiently and cost-effectively based on neutral diversity measures. However, this depends on the demographic history of the organism. It may be that this is an acceptable course of action only in bottlenecked, genetically depauperate populations, where drift has largely overridden selection. In our study, a handful (e.g. five) of the most polymorphic microsatellite loci would have allowed us to accurately determine the required number of founders to be translocated and to monitor the genetic diversity of the resulting populations. Further, using a suite of markers means that decisions are not made on a single functional region at the potential expense of other important regions (Radwan *et al.* 2010). Lastly, as next-generation sequencing technologies become cheaper and hence more accessible to conservation programmes, it should be possible to provide conservation managers with increasingly accurate information on genome-wide variation within and between populations to aid vital evidence-based decision making (Angeloni *et al.* 2012). The results presented here add to the growing evidence base on the impact and use of translocations as an important conservation tool and will, we hope, help inform translocation practices and the conservation of other endangered species.

3.6 References

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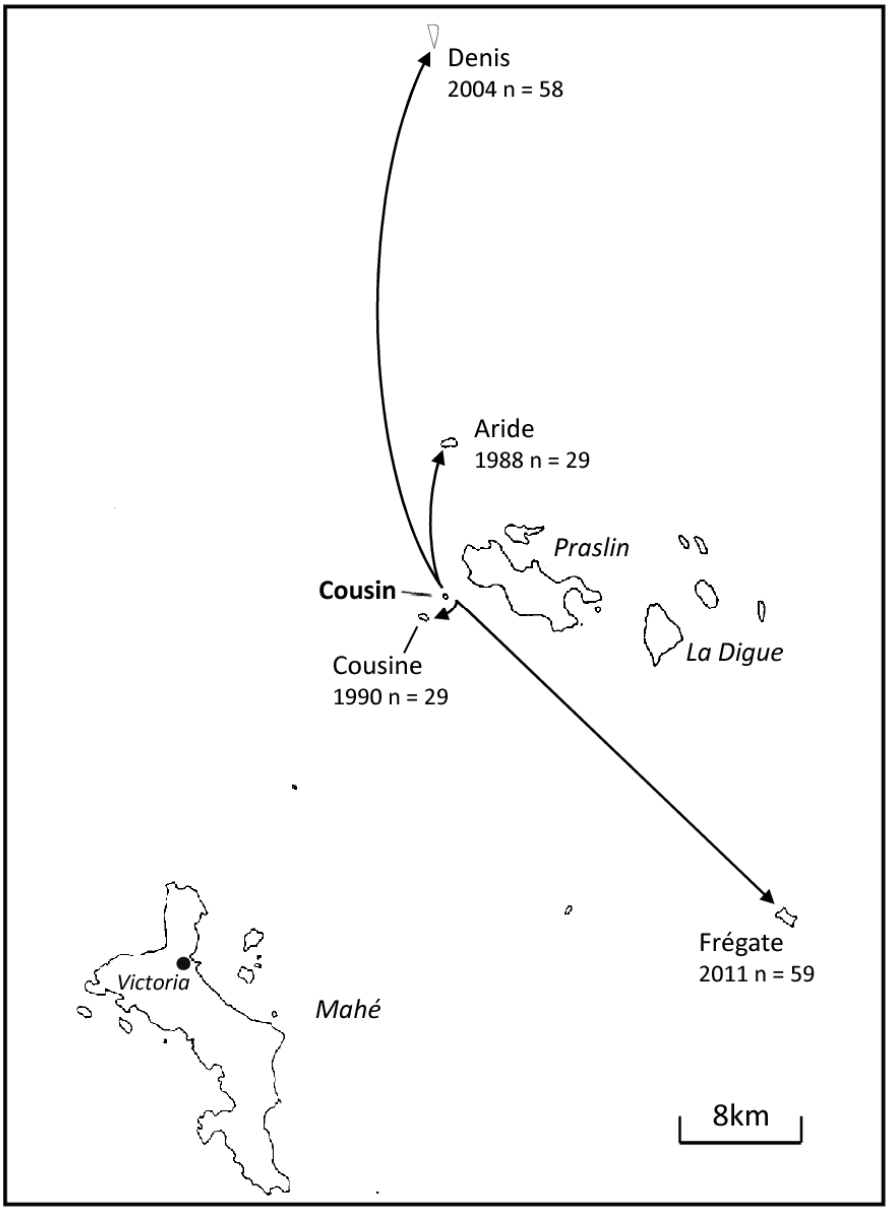
Supplementary Table S3.1 Null allele estimates for each of 30 microsatellite loci across each catch-year sample period for five island populations of Seychelles warblers where CN=Cousin, AR=Aride, CE=Cousine, DS=Denis and FR=Frégate. Estimates >0.1 highlighted in bold type.

Locus	CN 1993	CN 2005	CN 2011	AR 1993	AR 2005	AR 2011	CE 1997	CE 2005	CE 2011	DS 2004	DS 2011	FR 2011
Ase10	-0.04	-0.07	-0.05	-0.11	0.02	**0.24**	0.00	-0.08	0.04	-0.06	-0.09	-0.11
Ase13	-0.08	-0.03	-0.05	-0.12	0.01	-0.07	0.08	-0.06	0.03	0.06	-0.01	0.01
Ase18	-0.04	0.01	0.00	-0.04	-0.09	0.00	0.01	-0.04	-0.02	0.00	-0.09	-0.06
Ase25	-0.03	0.04	-0.01	-0.06	-0.07	-0.07	-0.04	-0.08	-0.08	-0.01	0.01	-0.03
Ase27	0.02	-0.02	-0.03	-0.03	-0.02	0.09	-0.11	**0.15**	-0.01	-0.05	0.01	-0.04
Ase35	0.01	0.07	0.00	-0.02	-0.06	0.02	-0.01	0.07	**0.17**	-0.04	0.07	0.00
Ase37	0.02	0.03	-0.02	-0.06	-0.05	0.10	0.02	-0.02	0.01	0.04	-0.04	-0.04
Ase4	-0.08	0.01	-0.07	**0.40**	-0.09	-0.07	0.10	-0.01	**0.16**	-0.03	0.01	0.02
Ase42	-0.11	-0.04	0.02	-0.12	-0.06	0.07	-0.05	**0.11**	0.07	-0.05	0.06	-0.02
Ase48	-0.02	-0.01	-0.10	-0.11	-0.05	-0.14	-0.03	-0.11	0.02	-0.03	0.06	-0.02
Ase56	-0.03	0.05	0.01	-0.08	-0.06	-0.05	-0.06	-0.04	-0.04	0.05	-0.01	**0.14**
Ase58	-0.02	0.02	-0.03	0.01	-0.10	-0.11	-0.05	0.06	-0.04	-0.04	0.00	-0.04
Ase6	-0.02	0.02	0.01	0.00	-0.04	-0.03	-0.04	-0.03	-0.07	-0.03	-0.09	0.04
Ase9	-0.06	-0.01	0.04	0.03	-0.13	-0.10	-0.17	0.04	-0.07	0.03	0.00	-0.03
Ase11	-0.09	0.00	0.01	-0.04	-0.12	-0.09	**0.20**	-0.03	0.07	-0.02	-0.08	**0.11**
Ase16	0.01	-0.01	0.02	-0.04	-0.06	0.01	0.05	-0.03	-0.07	0.01	-0.02	-0.05
Ase19	0.07	0.00	-0.04	-0.06	-0.07	**0.17**	0.08	-0.02	-0.05	0.01	0.08	0.00
Ase22	-0.13	0.09	-0.01	**0.11**	-0.03	-0.08	0.09	-0.08	0.09	0.01	**0.11**	0.09
Ase3	-0.12	-0.02	0.03	**0.10**	**0.21**	**0.14**	-0.02	-0.02	0.04	-0.01	**0.15**	0.02
Ase38	-0.04	0.00	-0.02	-0.23	0.00	-0.08	-0.16	0.03	0.04	-0.03	-0.12	**0.13**
Ase53	0.00	-0.04	0.06	**0.11**	-0.09	0.00	-0.06	0.05	0.06	0.06	-0.04	-0.03
Ase55-CEST	-0.03	0.06	-0.01	-0.03	-0.20	0.03	-0.03	0.05	-0.01	0.03	-0.07	-0.06
Ase61	0.05	0.06	0.04	-0.08	-0.01	**0.11**	0.00	-0.14	-0.12	0.00	-0.03	0.02
Ase64	0.03	-0.01	-0.03	0.02	-0.06	0.04	0.10	-0.07	-0.05	0.04	-0.12	-0.03
Ase7	-0.04	0.08	-0.05	-0.08	0.07	-0.04	0.00	0.01	0.00	0.02	-0.13	-0.07
Calex-08-Gga	**0.13**	0.00	-0.02	-0.11	-0.16	**0.11**	0.00	0.00	0.00	-0.04	0.03	-0.03
Cuμ4-Gga5	0.01	0.01	-0.09	-0.15	-0.02	-0.04	-0.12	-0.02	-0.07	**0.12**	0.02	-0.08
Pdoμ6	0.03	0.02	0.01	0.02	-0.06	0.02	0.00	0.03	-0.13	0.03	0.07	0.02
PmaTGAn42	0.03	0.00	-0.06	**0.31**	-0.07	0.09	0.00	0.04	-0.11	-0.07	-0.06	**0.12**
Pte24-CEST	0.00	0.00	0.00	0.00	0.00	-0.01	0.00	0.00	0.00	0.00	0.00	0.00

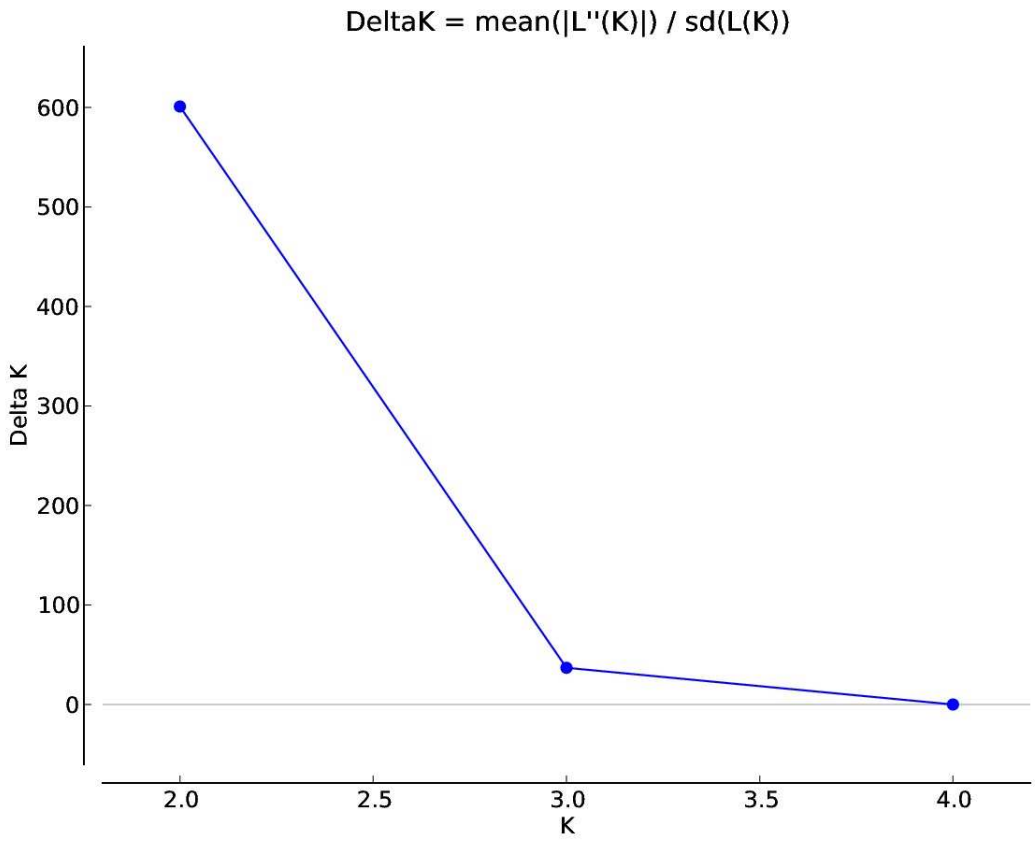
Supplementary Table S3.2 Pair-wise F_{ST} between each population sample across five island populations of Seychelles warblers. Microsatellites (lower) and major histocompatibility complex (upper) data.

	CN 1993	CN 2005	CN 2011	AR 1993	AR 2005	AR 2011	CE 1997	CE 2005	CE 2011	DS 2004	DS 2011	FR 2011
CN 1993		0.000	0.000	0.005	0.011	0.006	0.001	0.003	0.004	0.000	0.004	0.000
CN 2005	0.002		0.000	0.003	0.008	0.004	0.000	0.004	0.006	0.000	0.001	0.000
CN 2011	0.006	0.003		0.000	0.003	0.000	0.000	0.005	0.009	0.000	0.000	0.000
AR 1993	0.012	0.019	0.023		0.000	0.000	0.000	0.011	0.021	0.000	0.000	0.000
AR 2005	0.034	0.037	0.040	0.006		0.000	0.000	0.019	0.030	0.005	0.000	0.004
AR 2011	0.033	0.034	0.039	0.006	0.017		0.000	0.014	0.023	0.001	0.000	0.000
CE 1997	0.026	0.022	0.023	0.037	0.064	0.056		0.004	0.010	0.000	0.000	0.000
CE 2005	0.067	0.049	0.048	0.072	0.092	0.086	0.007		0.000	0.008	0.011	0.005
CE 2011	0.045	0.032	0.032	0.058	0.085	0.084	0.006	0.000		0.011	0.018	0.008
DS 2004	0.000	0.005	0.008	0.011	0.033	0.030	0.026	0.060	0.042		0.000	0.000
DS 2011	0.008	0.017	0.013	0.016	0.038	0.040	0.038	0.071	0.049	0.002		0.000
FR 2011	0.005	0.003	0.000	0.024	0.040	0.040	0.020	0.046	0.029	0.006	0.011	

Supplementary Figure S3.1 Map of the inner Seychelles archipelago with location, date and number of founding individuals for the four Seychelles warbler translocations. The source population Cousin is highlighted in bold. Longitude and latitude for each island is provided in the materials and methods of the article.



Supplementary Figure S3.2 Graph generated by STRUCTURE HARVESTER (Earl & vonHoldt 2012), displaying the change in ΔK against number of clusters (K) calculated following the method of Evanno *et al.* (2005), highlighting that K = 2 is the most likely number of genetic clusters across five island populations of Seychelles warbler.



Chapter 4

Translocation of the Seychelles warbler to Frégate Island, Seychelles.

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"…because they won't do it themselves!"

4.1 Abstract

In December 2011, 59 adult Seychelles warblers (*Acrocephalus sechellensis*), were captured on Cousin Island and translocated to Frégate Island using a hard release method. Frégate had been surveyed previously and identified as a suitable host for a substantial population of Seychelles warblers (*ca.* 500 currently rising to >2000 after habitat regeneration) based on our knowledge of the ecological requirements of this species. All birds survived the translocation and were released unharmed at the new site within 24 hours of capture. Close monitoring of both the new and source population was undertaken over a period of 18 months, including the ringing of all newly caught birds. By June 2013 a census revealed that the Frégate population had risen to 80 individuals. At this point 38 of the original 59 translocated birds were resighted, while 42 new birds hatched on Frégate were observed. There was also evidence that multiple generations had already hatched on the island. This evidence shows that the Seychelles warbler responded well to a hard release translocation, and the observed population growth on Frégate was comparable to previous warbler translocations. The source population on Cousin recovered to carrying capacity within a single breeding season (by June 2012). This is the fourth translocation of this species, completing the species action plan requirement for five populations of this endemic island passerine.

4.2 Background

The Seychelles warbler (*Acrocephalus sechellensis*) is a small, insectivorous passerine endemic to the granitic islands of the Republic of Seychelles. Due to habitat destruction for intensive coconut (*Cocos nucifera*) cultivation and the introduction of invasive predators (e.g. rats, *Rattus spp.*), the Seychelles warbler was extirpated from all islands except Cousin Island (4°20'S, 55°40'E, 0.29 km²) where the population may have fallen to as low as 26 individuals by the mid 20th century (Crook 1960; Chapter 2). The warbler has since been the focus of intensive conservation action that commenced with the purchase of Cousin in 1968 by a consortium led by ICBP (now BirdLife International). Since then the island has been managed solely for conservation and research. Careful habitat management, including the regeneration of native forest enabled the population to recover, and the population has remained stable at a carrying capacity of *ca.* 320 individuals since 1982 (Brouwer *et al.* 2006).

Although the Seychelles warbler is capable of sustained flight, inter-island dispersal is virtually absent (Komdeur *et al.* 2004) and so the species is unable to colonise new islands unaided. To increase the species range, translocations of 29 individuals were undertaken from Cousin to both Aride Island (4°12'S, 55°40'E, 0.68 km²) and Cousine Island (4°21'S, 55°39'E, 0.25 km²) in 1988 and 1990 respectively (Komdeur 1994). In 2001 Nature Seychelles commissioned a formal species conservation action plan for the Seychelles warbler. This plan outlined the objective of increasing this species range to five populations encompassing >3000 individuals in total to further enhance long term survival prospects and, concomitantly, potentially allow a downgrade in its conservation status to "Near Threatened" (Richardson 2001). At this point it was agreed that twice the number of individuals should be moved in future translocations to ensure more complete capture of the genetic variation present on Cousin Island within the new populations (Richardson pers. comm). Subsequently, a third translocation of 58 individuals from Cousin to Denis Island (3°48'S, 55°40'E, 1.42 km²) was undertaken (Richardson *et al.* 2006). The present work details the fourth translocation undertaken from Cousin to Frégate Island (4°35'S, 55°56'E, 2.19 km²) to fulfil the conservation action plan (Richardson 2001).

4.3 Action

4.3.1 Study site assessment

From 1999 to 2002, a general survey of 10 islands was undertaken by Nature Seychelles to assess their suitability for sustaining translocated populations of endangered endemic bird

species, including the Seychelles warbler (Hill 2002). Over three years prior to the translocation, the suitability of three selected islands was assessed specifically for the Seychelles warbler (D'Arros, Van de Crommenacker *et al.* 2009; North, Richardson & van der Woude 2010; Frégate, Richardson & Hammers 2011). Whole island surveys were conducted in which native and exotic vegetation was identified and its distribution mapped. Seychelles warbler prey availability was determined within this habitat mosaic using standardised insect counts following the protocols outlined by Komdeur (1992) and Brouwer *et al.* (2009). These data were used to estimate the quantity and quality of warbler habitat across each island. The complete absence of introduced predator species and the ongoing measures to prevent any possibility of accidental introduction were also confirmed at this point.

Frégate was selected to host the fifth Seychelles warbler population. The island is outside of the confirmed historic range of the species, however during the last glacial maximum the inner Seychelles islands would have been a single landmass. Furthermore a reconstruction of the species historic effective population size (N_e ≈ several thousand) suggests it was once abundant and widespread across these inner islands (Chapter 2). Frégate possesses 0.37 km^2 of suitable habitat and this is estimated to increase to 0.42 km^2 by 2016 as a result of the ongoing regeneration work being undertaken by the island proprietors. Food availability within this habitat was excellent, with prey counts higher than on Cousin (Richardson & Hammers 2011). Importantly, after the successful eradication of myna (*Acridotheres tristis*), Frégate was free of invasive predators (Millet & Shah 2001; Canning 2011). The survey estimated that Frégate would be able to currently sustain *ca.* 500 individuals and eventually >2000 after regeneration of habitat is completed (Richardson & Hammers 2011). An area of native pisonia (*Pisonia grandis*) woodland was identified as a suitable release site based on its close similarity to the preferred habitat on Cousin.

4.3.2 Pre-translocation census

As part of the ongoing research and monitoring undertaken by the Seychelles warbler Study Group we have detailed individual information on nearly all birds in the Cousin population (Komdeur 1992; Richardson *et al.* 2005; Barrett *et al.* 2013). However, prior to the translocation another complete island census was conducted from 25 November 2011 to 7 December 2011, to locate and check the suitability of individuals for translocation. The translocation was planned for December, prior to the minor breeding season (Komdeur 1991), when individuals are in peak condition (Richardson *et al.* 2006). A small percentage of territories may breed at any time of year and so the census enabled identification of

individuals exhibiting breeding behaviour that were subsequently excluded from catching effort. Cousin was used as the single source population for all translocated individuals as with all previous Seychelles warbler translocations. This was because 1) >96% of adult birds on Cousin are ringed (Richardson *et al.* 2001) so the identity, age and sex of most individuals is known, enabling us to target catching efforts towards the best founders, 2) the population was at carrying capacity and so many territories contained surplus, subordinate individuals (Brouwer *et al.* 2006) and 3) as the last remaining population, Cousin is the most genetically diverse and has not suffered any loss of variation as a result of earlier translocations (Chapter 3).

4.3.3 Capture

Based on previous experience and logistical feasibility, the translocation was split into two transfers; the first took place on 7 December 2011 and the second on 14 December 2011. For each transfer, four teams of 2-3 people were distributed around the island in allocated catching zones, where they were already familiar with the territory structure and the status of the occupying birds. These teams then relayed captured birds to a central processing team. Birds were caught using mist nets (5-6 nets of 9-12 m per team) and audio lures between 15:00 and 18:00 of the evening before, and from 06:30 and 13:00 on the day of transfer, at which point they were translocated. This timing was designed to allow the birds time (daylight hours) to feed both before capture, but also after release on Frégate, to ensure they were in the best condition for translocation and quick subsequent recovery of body weight. Individuals caught in the evening prior to the translocation were kept overnight in individual cardboard boxes (*ca.* 35 x 25 x 25 cm) furnished with a perch, pisonia leaves sprinkled with water and fresh termite (*Nasutitermes spp.*) eggs for food (Figure 4.1).

We attempted to catch individuals from across the entire island with only two stipulations; 1) avoid possibly breeding territories and, 2) avoid taking both dominant and subadults/subordinates/recent fledglings from the same territory, where they may be first order relatives. Subordinate individuals were particularly targeted as they were surplus, adult birds that were normally relatively young (but see Richardson *et al.* 2007), but often had breeding experience from helping the dominants in their natal territory (Komdeur 1996a). This makes them ideal founders for establishing a new population.

All individuals were checked for injuries and general body condition and their age and sex were checked in a central electronic database in order to select individuals fit for translocation and

to balance the sex ratio in the new population as much as possible. This was necessary as males are more responsive to the audio lures and thus more readily caught than females. Although genetic characteristics are known for many of the birds in this population (Richardson *et al.* 2002; Richardson *et al.* 2005) we did not use them to determine who should, or should not, be taken. Thus the translocation was blind in regards to genetic qualities. Any unringed birds were given a unique identifying combination of coloured leg rings and a metal British Trust for Ornithology (BTO) ring. Any faded or damaged rings were replaced. Standard measurements of wing and tarsus length and mass were recorded. A *ca.* 25 µl blood sample was taken from each bird by brachial venipuncture and stored in absolute ethanol. All birds were then placed individually into customised cardboard boxes (as above) ready for transportation (Figure 4.1).

Figure 4.1 Individual Seychelles warbler translocation boxes during preparation. Note small air hole punctures and pisonia (*P. grandis*) leaf lining. Occupied boxes were secured with duct tape.

4.3.4 Transport

A total of 59 birds (23 females, 36 males) were translocated to Frégate. Of these, 22 were transferred on 7 December and 37 on 14 December 2011, with 25 kept overnight and 34 caught on the day of transfer. The birds were transferred by helicopter; a journey time of 15 minutes. Some birds, caught shortly before departure, were therefore in captivity for less than 60 minutes.

4.3.5 Release

Prior to the translocated birds arrival, water was sprayed on the trees of the release site to provide drinking water. All birds were taken to the release site and immediately released within a 5 minute period. There was no mortality during the translocation and all individuals flew into the vegetation canopy without problem.

4.3.6 Post-release monitoring

The Frégate population was monitored for one week immediately after each release day. Cousin was monitored after 5 weeks (25 January 2012 to 28 February 2012) and Frégate was monitored again after 8 weeks (15 to 28 February 2012). Extended monitoring of Cousin was undertaken from June to October 2012 and of Frégate from March to June 2013. All monitoring was undertaken by teams of 2-3 people. Censuses were conducted using transects across the islands in which birds were located by a variety of methods; "phishing" (blowing harshly through closed lips to create a scolding-type vocalisation), whistling and/or using song playback, listening for the song, distinctive bill-snap produced when the warbler is feeding, or the begging calls of chicks and dependant fledglings. Once located, individuals were followed for a minimum of 15 minutes to determine their territory boundaries, breeding status and any interactions with other warblers. We attempted to catch all unringed birds using mist nets and song playback on both islands. Any unringed individuals were given a unique indentifying combination of coloured leg rings and a metal British Trust for Ornithology (BTO) ring. All birds were processed as described previously.

4.4 Consequences

4.4.1 Frégate population

A total of 50 (85%, 20 female, 30 male) translocated individuals were resighted during February 2012 (8 weeks post-release) in 28 established territories. Nineteen pairings were confirmed and two breeding attempts were recorded. The birds had colonised many areas across the island, with individuals sighted on the opposite side of the island to the release site (Wright & Richardson 2012). Both sexes showed high annual survival (males 0.92 ± 0.04, females 0.95 ± 0.03, Teunissen 2013). By June 2013, the total census had increased to 80 warblers with 33 defined territories, an overall population growth of 36% - though a small number of unringed individuals hatched on Frégate makes an exact figure difficult to ascertain. Of the 59 founders, 38 were resighted during this census, with a lower annual survival than for 2012 (males 0.85 ± 0.07, females 0.77 ± 0.07, Teunissen 2013). A minimum of 42 individuals had hatched on Frégate, six of which were the begging offspring of birds hatched on Frégate

themselves (Teunissen 2013), indicating at least two generations since translocation. The highest density of territories was around the release site, in the areas of habitat considered highest quality for warblers (Richardson & Hammers 2011). However, the warblers were often observed using habitat consisting of mostly introduced tree species such as cinnamon (*Cinnamomum spp.*), cashew (*Anacardium spp*). and coco plum (*Chrysobalanus* spp.; Wright & Richardson 2012) that had been designated as unsuitable (Richardson & Hammers 2011). As expected, no territories were found within areas dominated by coconut.

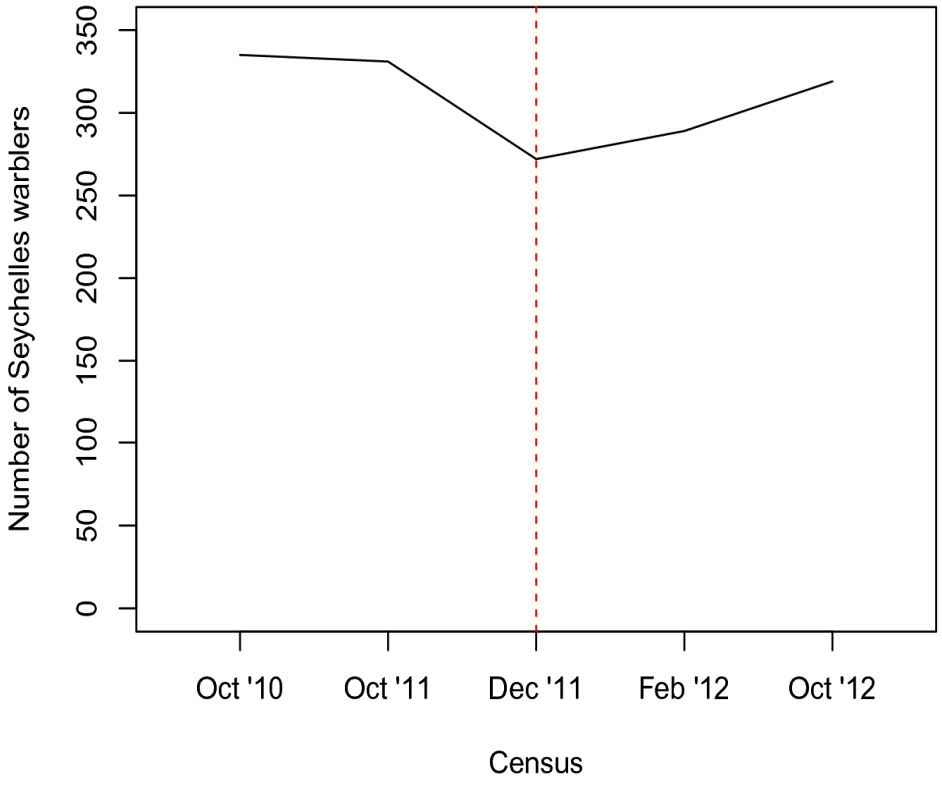

Figure 4.2 The Cousin Seychelles warbler population size (ringed individuals) at the end of each main breeding season (October), immediately following translocation (December 2011, dashed line) and during two post-release monitoring periods.

4.4.2 Cousin population

The post-release census of Cousin (January – February 2012) revealed a total of *ca.* 290 ringed individuals (Figure 4.2). Around 25% of all territories were engaged in breeding activity during the minor breeding season (December – February). A total of 23 territories had one or more dominant individuals removed during the translocation. By February 2012, 14 of these 23 territories had acquired a new dominant breeder, 3 contained a single individual in a reduced size territory, 3 had ceased to exist and a further 3 could not be determined. By October 2012, the population census was again *ca.* 320 ringed birds (Figure 4.2). Monitoring of both populations is ongoing.

4.5 Discussion

The translocation of warblers to Frégate completes the requirement for the establishment of five populations of Seychelles warblers. Three of these populations (Cousin, Aride and Cousine) are already at carrying capacity (Brouwer *et al.* 2009). The Frégate population is showing growth similar to previous translocations (Richardson *et al.* 2006). It is encouraging that Seychelles warblers on Frégate were readily utilising habitat deemed unsuitable during earlier surveys. This suggests that the upper population size estimate of >2000 may be exceeded.

Unusually for an avian translocation programme, no mortality occurred during this or any of the preceeding translocations of Seychelles warblers, and survivorship in the establishing populations was high (Teunissen 2013). A study by Taylor (2006) found that time in captivity affected the probability of mortality in translocations of saddlebacks (*Philesturnus carunculatus*). Here we applied a 'hard release' with minimum time in captivity, which appears to be a very successful method for the Seychelles warbler. This species may be particularly resilient to stress and thus suited to a hard release method. We emphasise the invaluable contribution of several years of comprehensive planning and surveying prior to the translocation. This enabled us to understand the species and informed the selection of the best new island/habitat and which founders to take to maximise survival and population establishment.

The Frégate translocation was largest of the four translocations, with 59 founders. Recent work comparing genetic diversity between Seychelles warbler populations shows that this was a sufficient number of individuals to maintain the levels of genetic diversity observed in both neutral and adaptive immune genes present in the source population on Cousin (Chapter 3).

The translocation of 59 Seychelles warblers from Cousin reduced its population size by *ca.* 20%. We subsequently observed rapid growth of this population through the course of the minor (November – March) and following main (June – September) breeding seasons, with the population recovered to carrying capacity by October 2012, less than one year after the translocation took place. That 25% of territories bred during the minor breeding season, is as expected based on long term monitoring of this population (Komdeur 1996b). The rapid repopulation of Cousin is, therefore, more likely due to increased survival of offspring than an increase in breeding *per se*. Overall the impact on the source population has been minimal.

Although the last two populations established on Frégate and Denis are not yet near reaching saturation, all five populations of the Seychelles warbler do now appear to be well established. The total population of Seychelles warblers is now estimated at *ca.* 2800 adults; Cousin = 320 (Brouwer *et al.* 2006), Cousine = 210 (Van de Crommenacker & Richardson 2007), Aride = 1850 (Orchard 2004), Denis = 300 (J. van der Woude, unpublished data) and Frégate = 80 (Teunissen 2013). As the newer populations grow to capacity we expect the total warbler population to eventually exceed 7000. These numbers, spread as they are across five separate islands, may then justify this species being downgraded to 'near threatened' (BirdLife International 2014). The monitoring of all Seychelles warbler populations will continue into the foreseeable future, hand-in-hand with research on this species that has become a model for conservation, evolutionary and ecological research.

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Chapter 5

Experimental investigation of MHC-dependent social mate choice in Seychelles warblers

A version of this manuscript has been submitted for publication in *Behavioral Ecology*

A female incubates her egg, ever alert for fodies and skinks

5.1 Abstract

The prevalence and significance of pre-copulatory mate choice remains keenly debated. The major histocompatibility complex (MHC) plays a key role in vertebrate adaptive immunity, and variation at the MHC influences individual survival. Although MHC-dependent mate choice has been documented in certain species, many other studies find no such pattern. This may, at least in part, be because in natural systems constraints may prevent the optimisation of mate choice and confound patterns of underlying preference. We used translocations to previously unoccupied islands to experimentally reduce constraints on female social mate choice in the Seychelles warbler (*Acrocephalus sechellensis*), a species in which patterns of MHC-dependent extra-pair paternity, but not social mate choice, have been observed. Contrary to expectations, we find no evidence of MHC-dependent social mate choice in the new populations. Instead, we find that male pair status (paired vs. unpaired) is better predicted by male age and heterozygosity. These effects were not observed in the source population, indicating that constraints there inhibited pairing patterns. Our research confirms that female Seychelles warblers do not use MHC-dependent social mate choice to increase fitness. It also suggests that contemporary constraints may not be the reason behind the lack of MHC-dependent mating patterns in other species.

5.2 Introduction

The prevalence and significance of pre-copulatory mate choice remains a keenly debated topic in sexual selection, mainly due to difficulties in quantifying the evolutionary costs and benefits of being 'choosy' (Andersson 1994; Kokko *et al.* 2003). The optimisation, within offspring, of genetic variation at functionally important genes, such as the major histocompatibility complex (MHC), is one potential indirect benefit that has received a lot of attention (Yamazaki *et al.* 1976; Jordan & Bruford 1998; Milinski 2006). The MHC plays a central role in antigen recognition in the adaptive immune response of vertebrates (Klein 1986). As different MHC genotypes confer differential pathogen resistance and, therefore, fitness to individuals (Briles *et al.* 1977; Wedekind *et al.* 2004), mechanisms may have evolved to optimise the MHC genotypes that offspring inherit (Penn & Potts 1999). This logic makes the MHC an obvious candidate as the genes that could underpin indirect benefits of mate choice.

Under the 'good genes' model of sexual selection, individuals choose partners based on indicators of condition, such as secondary sexual traits (Hamilton & Zuk 1982) that can be influenced by MHC characteristics (Ditchkoff *et al.* 2001). By choosing a mate with a superior phenotype, individuals can obtain genetic benefits by increasing the MHC diversity of their offspring and/or by providing them with specific advantageous alleles (Hamilton & Zuk 1982). Under the 'compatibility' hypothesis, individuals choose between potential mates to obtain an optimal level of MHC diversity in offspring. What constitutes a good mate depends on the complementarity of the maternal and paternal genotypes (Yamazaki *et al.* 1976; Milinski 2006). Both models are normally viewed from the female perspective (but see Gillingham *et al.* 2009; Edward & Chapman 2011). Finally, MHC genes may also act as markers of relatedness and be used to avoid inbreeding (Brown & Eklund 1994; Penn & Potts 1998). Numerous studies have investigated MHC-dependent mate choice with evidence accumulating for both 'good genes' and 'compatibility' based mechanisms (e.g. Penn & Potts 1999; Kokko *et al.* 2003; Andersson & Simmons 2006; Milinski 2006; Kotiaho & Puurtinen 2007; Løvlie *et al.* 2013). However, other studies find no evidence of MHC-dependent mating patterns (e.g. Paterson & Pemberton 1997; Westerdahl 2004; Huchard *et al.* 2010).

Mate choice can manifest in social patterns, such as choosing of a social mate, or in genetic patterns, such as bias in offspring MHC genotypes (Jennions & Petrie 2000; Consuegra & Garcia de Leaniz 2008). An absence of MHC-dependent mate choice may be due to constraint on choice, which may occur to some extent in almost all species (Arnqvist & Rowe 2005) due a variety of factors including social monogamy (Cohas *et al.* 2006), intra-specific competition

(Wong & Candolin 2005) and forced pairings (Casalini *et al.* 2009). Significant research has focussed on the consequences of constraints for the evolution of alternative mating strategies such as reproductive compensation and promiscuity (Cohas *et al.* 2006; Gowaty *et al.* 2007; Setchell & Huchard 2010), but the implications of mate choice constraints for MHC diversity in wild populations remains unclear.

Here, we take an experimental approach to investigate whether the removal of constraints leads to the expression of MHC-dependent social mate choice in the Seychelles warbler (*Acrocephalus sechellensis*). This socially monogamous species was previously restricted to a single island, Cousin, where the population has been at carrying capacity since 1982 (Brouwer *et al.* 2009). On Cousin, a combination of habitat saturation, longevity and social fidelity is thought to constrain social mate choice (Richardson *et al.* 2005). However, Seychelles warblers are highly promiscuous, with extra-pair paternity (EPP) accounting for *ca.* 40% of offspring (Richardson *et al.* 2001). This promiscuity is linked to MHC variation: females are more likely to gain EPP if their pair male is of low MHC diversity and do so with extra-pair males that are more MHC-diverse (Richardson *et al.* 2005), consequently improving the survival of their offspring (Brouwer *et al.* 2010). Translocations to two new islands were undertaken as part of the long-term conservation of this species (Richardson *et al.* 2006; Chapter 4). Significantly more males than females were translocated to each island, where a surfeit of optimal habitat allowed each male to establish a high quality territory without constraints. Consequently females had the opportunity to choose between multiple males, all with high quality territories. Thus we provide a relatively unconstrained arena in which MHC-dependent female social mate choice could be expressed. Given the offspring survival benefits resulting from mating with a MHC diverse male, we expect MHC to play an important role in unconstrained social mate choice. Under the 'good genes' hypothesis we expect females to prefer males with higher MHC diversity. Under the 'compatibility' hypothesis, the MHC dissimilarity between pairs will differ from that expected under random mating, with females pairing with maximally or optimally (Milinski 2006), MHC dissimilar males. Finally we test whether stability of the initial pair bonds is MHC-dependent. If 'compatibility' is important, pairs that divorce are expected to be more MHC-similar than faithful pairs.

5.3 Materials & Methods

5.3.1 Study populations

Each translocation was performed as outlined by Richardson *et al.* (2006), with all birds caught on Cousin (4°21'S 55°38'E, 0.29 km², Figure 5.1). Translocation of existing pairs was avoided,

although a small number were transferred (see results). However, previous studies showed that birds paired in the source population did not re-pair in the new populations (Komdeur 1996). Individuals were identified with a unique combination of coloured leg rings. A (*ca.* 25 µl) blood sample was taken from each bird by brachial venipuncture and stored in absolute ethanol. A total of 58 birds (34 males, 24 females) were moved to Denis (3°48'S 55°40'E, 1.42 km²) in 2004 and 59 birds (36 males, 23 females) to Frégate (4°35'S 55°56'E, 2.19 km²) in 2011 (Figure 5.1). Each translocation was undertaken in two batches on different days (Denis: n = 35 on 30/05/2004, n = 23 on 12/06/2004, Frégate: n = 22 on 07/12/2011, n = 37 on 14/12/2011). All individuals were released at the same site on each island. Age at translocation (determined in reference to lay year estimated at first capture on Cousin), was classified into 'young' (<2 years) and 'old' (>2 years), based on Brouwer *et al.* (2006). There was no bias in age of individuals in each batch and catches were undertaken immediately prior to translocation. Each new population was censused and monitored for breeding up to three months post release (Denis: continually from release until August 2004, Frégate: February 2012) and again after one year post-release (Denis: May–August 2005, Frégate: March–May 2013).

5.3.2 Social mate choice

Territories were mapped on each island. The average territory size of Seychelles warblers on Cousin is 250 m^2 (Ridley *et al.* 2004). There was at least 300 000 m^2 of suitable habitat on Denis (Richardson *et al.* 2006) and 390 000 m^2 on Frégate (Richardson & Hammers 2011). Thus there were no spatial or habitat quality constraints for males. Individuals were located and followed for a minimum of 15 minutes repeatedly during the field period, in which interactions with other individuals were recorded. After a pair bond and a territory have been established Seychelles warblers display a specific repertoire of behaviours that allows clear classification of pair status (Komdeur 2001). Previous studies found that although a few pairs may form and nest within days of translocation, most stable pairings only formed after two months of rapid switching (Komdeur 1996). Hence social mate choice was assessed after three months and by multiple observations where possible. Pairings were reassessed one year after the initial social pairing observations. Divorce was identified when both individuals were re-sighted, with at least one individual in a new pair. In cases where one individual of a pair was not re-sighted over consecutive fieldwork periods, it was assumed that individual had died and the pairing was considered faithful until death. Any subsequent pairing of the surviving individual was not considered the result of divorce and was excluded from analysis, but we acknowledge this is a conservative measure.

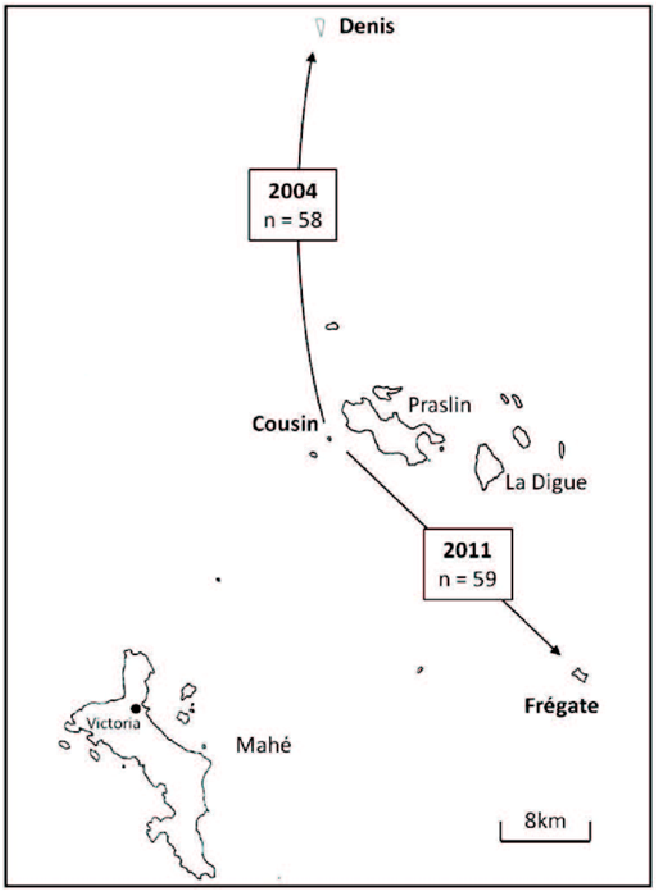

Figure 5.1 Map of the inner Seychelles Islands, showing the two translocated Seychelles warbler populations of Denis and Frégate with year and number of individuals transferred.

5.3.3 Molecular analyses

Samples were genotyped at 30 microsatellite loci following chapter 2 as part of the long-term project. We tested for deviations from Hardy-Weinberg equilibrium and for linkage disequilibrium between loci in each population using GENEPOP v. 4.1 (Raymond & Rousset 1995). Null allele frequencies were estimated using CERVUS v. 3.0 (Marshall *et al.* 1998). Three estimators of pairwise relatedness *r* (Queller & Goodnight 1989; Lynch & Ritland 1999; Wang 2002) were calculated in COANCESTRY v. 1.0 (Wang 2011). These three estimators were highly correlated (Mantel tests, all $r \geq 0.80$). Results remained consistent regardless of the *r* estimate used, therefore only Queller & Goodnight's *r* is reported.

Variation at exon 3 of MHC class I (which codes for the peptide binding region (PBR) involved in antigen recognition) was screened using reference-strand mediated conformation analysis (RSCA) and the primers from Richardson and Westerdahl (2003), following the method of

Worley *et al*. (2008). Each segregating RSCA variant corresponded to a unique 255bp amino acid coding sequence (Richardson & Westerdahl 2003). A total of ten MHC class I variants have been detected in the Seychelles warbler using this method, with individuals possessing between 2-8 variants each, suggesting that at least four class I loci are amplified (Richardson & Westerdahl 2003). Although it is impossible to identify which locus each variant comes from they are hereafter termed 'alleles' for simplicity. This method does not provide a measure of locus-specific heterozygosity, but an overall estimate of MHC Class I exon 3 diversity, shown to be an important parameter linked to fertilisation patterns and survival in the Seychelles warbler (Richardson *et al.* 2005; Brouwer *et al.* 2010). The codons comprising the PBR were superimposed onto the Seychelles warbler sequences (see Richardson & Westerdahl 2003).

MHC-dependent mate choice could be based upon positively selected sites (PSS), which may differ from the superimposed PBR. To identify PSS, MHC Class I exon 3 sequences from a range of passerine genera were downloaded from NCBI GenBank; *Acrocephalus* (n = 16)*, Passer* (n = 38)*, Parus* (n = 64, of which *Cyanistes* = 59) and *Carpodacus* (n = 28) and aligned to the Seychelles warbler (n = 10) in BIOEDIT v. 7.1 (Hall 1999). Three methods were employed to detect positive selection. Single likelihood ancestor counting (SLAC) and fast unbiased Bayesian approximation (FUBAR) are maximum likelihood methods which estimate the non-synonymous to synonymous substitution rate (dN/dS ratio, ω) at each codon, the latter utilising an Monte Carlo Markov-chain approach to increase model accuracy (Kosakovsky Pond & Frost 2005; Murrell *et al.* 2013). The third method – the mixed effects model of evolution (MEME) - identifies episodic bouts of positive selection across an alignment by allowing ω to vary by codon and branch within the phylogeny (Murrell *et al.* 2012). Each method was implemented under the conservative general time reversible model and neighbour-joining tree with strict probabilities of < 0.05 (SLAC and MEME) and posterior probability of ≥ 0.95 (FUBAR) using HYPHY (Kosakovsky Pond *et al.* 2005) and a web-based user interface operating on a remote cluster available at http://www.datamonkey.org (Delport *et al.* 2010). Only codons identified by all three conservative methods were accepted as putatively PSS.

5.3.4 Statistical analyses

Statistical analyses were performed in R v. 2.15 (R Development Core Team 2012) unless otherwise stated. Throughout, the term 'pairs' denotes observed pairings, and 'dyads' all other possible male-female pairs artificially constructed. Comparisons of pairs and dyads were performed using randomisation tests (Manly 1997) in MSEXCEL plug-in POPTOOLS v. 3.2 (Hood 2010). In each instance, data were re-sampled without replacement and tested 10^5 times.

Estimates of significance were calculated as the proportion of repetitions in which the resampled ANOVA *F* value was equal to, or larger than, the test ANOVA *F* value. To control for any effect of inbreeding avoidance on social mate choice, as well as comparing MHC, we also compared relatedness (*r)* of pairs and dyads for each island separately using ANOVA. To investigate 'good genes' predictions, the probability of a male being paired was analysed using a generalised linear model with a binomial error structure and logit link function, with number of MHC alleles and standardised individual heterozygosity (Hs_exp) as continuous variables and island and age class as categorical variables. Data from both islands were combined to maximise sample size, hence inclusion of 'island' in the model. The R package GENHET (COULON 2010) was used to calculate Hs_exp. The maximum model is analysed and reported. MHC similarity between pairs/dyads was calculated in two ways. First, the proportion of alleles shared (S_{xy}), which is double the number of alleles shared between two individuals, divided by the sum of each individual's alleles [$S_{xy} = 2N_{xy}/(N_x + N_y)$] (Wetton *et al.* 1987). Second, amino acid divergence (p-distance) was calculated between each MHC allele sequence for codons (1) involved in the PBR and (2) identified as putative PSS. Amino acid p-distances were calculated in MEGA v. 5.1 (Tamura *et al.* 2011). The mean pairwise amino acid divergence between pairs/dyads was then calculated as above. Under the optimality hypothesis observed pairs may have a comparable intermediate level of MHC similarity to random pairings but are predicted to show less variation around this mean, therefore we tested variance in MHC similarity of observed vs. simulated pairs using the same randomisation approach. The association between the MHC similarity of a pair and the likelihood of divorce was tested using Mann-Whitney U tests on divorced vs. faithful pairings, using each measure of MHC similarity.

5.4 Results

All individuals were released unharmed in both translocations. Three months post-release, 56 birds (97%) were resighted on Denis and 50 birds (85%) on Frégate. Annual survival of adult Seychelles warblers is exceptionally high (Brouwer *et al.* 2006) but unpaired birds are more difficult to locate than paired birds due to aggressive territoriality in the latter. All but one individual was resighted on Denis in subsequent years and we assumed similar survival on Frégate (for this reason, individuals not seen during the initial study period (Denis = 2, Frégate = 9) were treated as alive and unpaired). A total of 40 pairings was confirmed in the two translocated populations: 40 males were considered paired and 30 unpaired. MHC data were unavailable for three individuals (Denis = 2, Frégate = 1) and these were excluded from analyses along with their pair bird, leaving 37 pairings (Denis = 19, Frégate = 18). Eight existing pairs from Cousin were translocated (Denis = 7, Frégate = 1) but only two of these pairs re-

paired once released (both on Denis). Observations of pairs one year after the initial monitoring revealed that 29 pairs had remained faithful and eight had divorced.

5.4.1 Genetic markers

Microsatellite genotypes were compiled for all 111 individuals included in the analyses (Denis = 54, Frégate = 57). Neither population showed significant departure from Hardy-Weinberg equilibrium at any locus. Linkage disequilibrium was detected between one loci pair in Denis (*Ase*-13 and *Ase*-48, P_{crit} = 0.0001) and between two loci pairs in Frégate (*Ase*-56 and *Ase*-38, *Ase*48 and Cuu4-gga, both P_{crit} = 0.0001) after Bonferroni correction. Null allele frequencies of 0.11 were detected at loci *Ase*-56 and *Ase*-38 in the Frégate population, but -0.013 and -0.096 respectively in the Denis population. Analyses repeated with and without one of each pair of loci showed no qualitative difference (data not shown) and so all loci were retained.

There was no difference in the number of MHC alleles in each sex (females = 4.70 ± 0.22, males = 4.61 ± 0.17, $T_{89.282}$ = -0.33, P = 0.75), or between islands (Denis = 4.78 ± 0.18, Frégate = 4.53 ± 0.20, $T_{108.289}$ = -0.92, P = 0.36). Selection tests indicated that seven of the 85 codons of exon 3 were putatively PSS, of which three corresponded to the 7th, 9th and 11th codons of the superimposed PBR (Figure S5.1).

Table 5.1 Logistic regression model predicting the pairing status of male Seychelles warblers within the newly established populations in relation to MHC diversity, age class (young or old), individual standardised heterozygosity (Hs_exp) and island (Denis or Frégate). Maximum model χ^2 = 10.59, P = 0.03 and R^2 = 0.20 (Nagelkerke). Parameters significant at P < 0.05 are given in bold.

Predictors	β (SE)	Wald	*df*	95% CI for odds ratio			*P* value
				Lower	Odds ratio	Upper	
Constant	*-2.49 (1.72)*	*-1.45*	*1*	*0.002*	*0.08*	*2.26*	*0.15*
MHC diversity	-0.19 (0.19)	-0.98	1	0.56	0.83	1.20	0.32
Age class	**1.23 (0.57)**	**2.23**	**1**	**1.20**	**3.55**	**11.38**	**0.02**
Hs_exp	**2.95 (1.46)**	**2.02**	**1**	**1.23**	**19.16**	**413.10**	**0.04**
Island	0.03 (0.56)	0.05	1	0.34	1.03	3.22	0.96

5.4.2 Social mate choice

Inbreeding avoidance: pairwise relatedness (*r*) varied between *r* = -0.31 and 0.41 (mean ± SE = 0.02 ± 0.03) across both populations with five pairs exhibiting *r* > 0.25. No difference was detected between the pairwise relatedness of pairs vs. dyads on either island (Denis; dependent *F* ≥ test *F* in 40436/10^5 iterations, *P* = 0.40, Frégate; dependent *F* ≥ test *F* in 15906/10^5 iterations, *P* = 0.16).

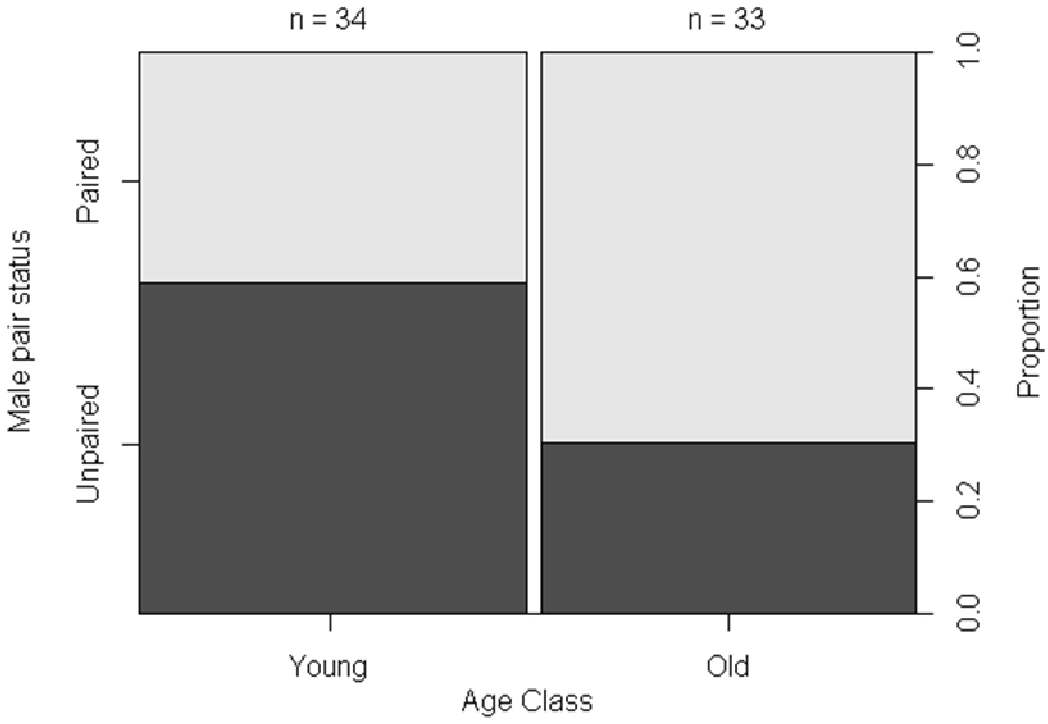

Figure 5.2 Proportions of male Seychelles warblers paired (light grey) and unpaired (dark grey) in the newly established populations for each age class, after three months post-release: young (< 2 years, n = 34) and old (> 2 years, n = 33). Data are combined from both translocated populations.

Good genes: the full generalised linear model was a significant improvement upon the null model (χ^2 = 10.59, *P* = 0.03). No collinearity was detected between predictors (all tolerances ≥ 0.88) and there was no difference in Hs_exp between age classes ($T_{64.83}$ = 0.02, *P* = 0.98). MHC diversity did not predict whether males were paired or not (Table 5.1). Age class and, to a lesser degree, individual neutral heterozygosity (Hs_exp), were both significant predictors of male pair status. Older males were 3.5 times more likely to be paired than younger males (Figure 5.2) and more heterozygous males were more likely to be paired than less heterozygous males. Including which transfer batch (date) individuals were moved, or testing for a quadratic effect of male MHC diversity did not influence the effects of age and heterozygosity on male pairing.

MHC compatibility: there was no difference in mean MHC allele sharing (S_{xy}) between pairs vs. dyads on either island (Denis $P = 0.95$, Frégate $P = 0.22$). There was no difference in variance between observed and simulated pairs (Denis $P = 0.87$, Frégate $P = 0.28$). Similarly, there was no difference in mean p-distance between pairs vs. dyads for either PBR codons (Denis $P = 0.69$, Frégate $P = 0.19$) or PSS codons (Denis $P = 0.89$, Frégate $P = 0.14$).

Divorce: no difference was detected between divorced vs. faithful pairs with any of the measures of MHC similarity; S_{xy} (divorced [median = 0.59] vs. faithful [median = 0.67] $W = 128$, $P = 0.66$), PSS p-distance (divorced [median = 0.26] vs. faithful [median = 0.38] $W = 165.5$, $P = 0.07$) or PBR p-distance (divorced [median = 0.33] vs. faithful [median = 0.35] $W = 161.5$, $P = 0.10$).

5.5 Discussion

We found no evidence that social pairing or divorce in founding populations of Seychelles warblers were influenced by male MHC diversity or male-female MHC compatibility. However, we found an effect of male age; older males were 3.5 times more likely to obtain a partner than young males. We also found that males that were more heterozygous across a suite of putatively neutral microsatellite markers were more likely to be paired than less heterozygous males. These effects were not observed in the source population (Richardson *et al.* 2005), indicating that constraint reduction resulted in the expression of previously inhibited patterns of social pairing, albeit non-MHC-dependent.

Previous work on Cousin found no evidence for MHC-dependent social mate choice, but did find strong evidence of MHC-dependent EPP (Richardson *et al.* 2005) and survival (Brouwer *et al.* 2010). Given that MHC diversity appeared to be so important, it was suggested that the lack of social mate choice was due to constraints on breeding opportunities imposed by habitat saturation, fidelity and longevity (Richardson *et al.* 2005). However, we still found no evidence of MHC-dependent social mate choice where the constraints on female choice were greatly reduced (if not totally removed). Females did not choose more MHC diverse males over less diverse males and the MHC dissimilarity between pairs did not differ from that expected under random mating. Assessing MHC dissimilarity based on presence/absence coefficients, such as S_{xy}, overlooks the functional differences between alleles on which choice may be based (Schwensow *et al.* 2008). Consequently we also analysed pairings using amino acid divergence of the PBR and PSS. However, we still found no evidence for MHC-dependent patterns of social mate choice.

Many hypotheses have been put forward to explain why divorce occurs within socially monogamous species (Rasmussen 1981; Choudhury 1995; Perez-Staples *et al.* 2013). One hypothesis is that failure of reproductive attempts may weaken the pair bond and drive divorce (Rasmussen 1981), though this is debated (Ihle *et al.* 2013). In the population of Seychelles warblers on Cousin, divorce can, occasionally, occur where experienced birds leave inexperienced birds after unsuccessful reproductive attempts (Komdeur 1996) but most pairs remain socially faithful until the death of one individual. However, as in initial pairings, divorce may be limited by constraints on opportunities to find a new available partner/territory in this saturated population. Here we specifically tested whether divorce was MHC-dependent in these new unconstrained populations. We found little evidence that divorce was related to the MHC characteristics of the pair birds. A slight trend was observed between divorce and PSS ($P = 0.07$) that suggests pairs with more similarity at PSS were more likely to divorce than those more different at PSS. However, although this is intriguing we acknowledge that statistical power and further inference is limited by the sample size.

Various reasons might have prevented us from finding an effect of MHC on mating patterns. For instance, inbreeding avoidance may mask MHC-dependent choice. However, previous work on the Cousin population found no evidence of inbreeding avoidance in this species (Richardson *et al.* 2004; Eikenaar *et al.* 2008), an unusual finding for a cooperatively breeding species (Jamieson *et al.* 2009). Furthermore our present results show that even after removing the constraints on a female's freedom to choose a social partner, relatedness did not influence choice. In fact, the relatedness of five pairs exceeded half-sibship ($r > 0.25$) suggesting little attempt to avoid inbreeding, and so this is unlikely to have confounded our MHC analyses.

Subtle patterns of MHC-dependent social mate choice may not have been detected owing to a limited sample size (n = 37 pairs). However, similar samples sizes have detected MHC-dependent patterns in other studies of wild populations (Bonneaud *et al.* 2006; Juola & Dearborn 2012) and we do still find effects of age and heterozygosity in this study. Another issue is that recombination can reduce the accuracy of likelihood selection methods (Anisimova *et al.* 2003) and thus the identification of PSS. Complex recombination (e.g. gene conversion) can occur at the MHC region (Wittzell *et al.* 1999; Spurgin *et al.* 2011). Therefore we acknowledge that the conservative tests employed here could have misidentified or missed PSS thus weakening our ability to detect choice based on these sites. Finally, we may not have screened the class of MHC on which choice may be based, for instance Strandh *et al.* (2012) identified 'compatibility' driven mate choice in blue petrels (*Halobaena caerulea*) at MHC class

IIB, but not at class I, loci. However, in the Seychelles warbler, MHC class I dependent patterns of EPP have been detected (Richardson *et al.* 2005) and screening class IIB revealed an almost complete lack of diversity (Hutchings 2009). Overall, an absence of MHC-dependent social mate choice, even when choice constraints have been much reduced, is therefore the most likely explanation of our findings. These results concur with those of the congeneric great reed warbler (*Acrocephalus arundinaceus*; Westerdahl 2004).

We observed a weak effect of neutral heterozygosity on the pairing status of males, suggesting that more heterozygous males are more likely to be paired than less heterozygous males. Further, the lack of inbreeding avoidance suggests that male-female complementarity does not play a role in this bias. Mate choice under a "good-genes-as-heterozygosity" model has been widely reported (Kempenaers 2007) and may provide an explanation for this finding. Unfortunately sample size limits further interpretation and obtaining larger scale experimental datasets in this species is not feasible.

Our study finds evidence that older males (>2 years) were 3.5 times more likely to be paired than younger males (<2 years), even though Seychelles warblers can breed successfully at eight months of age (Komdeur 1992). The lack of collinearity between model predictors along with no difference in heterozygosity between age classes, suggests that the age effect is not a result of female choice for more neutrally heterozygous individuals or vice versa, or that older males were more MHC diverse. Age-dependent social mate choice has been observed in many other species (Kokko & Lindstrom 1996; Kokko 1998), and extra-pair fertilisation success is also age-dependent in various species (e.g. Wetton *et al.* 1995; Richardson & Burke 1999; Tarof *et al.* 2012). However, active female mate choice is only part of sexual selection and mechanisms such as male-male competition, sperm competition and cryptic female choice can also provide genetic benefits (Andersson 1994; Jennions & Petrie 2000; Kotiaho & Puurtinen 2007; Løvlie *et al.* 2013). Determining whether our findings are a result of active female choice for older and more heterozygous males, or male-male competition is difficult. Seychelles warbler territory acquisition is age-related, with older males more likely to gain a breeding territory than younger ones, probably mediated by male-male competition (Eikenaar *et al.* 2009). However, there was no competition for high quality territories in these translocated populations. It is plausible that older males were more successfully able to compete for females, perhaps through other forms of male-male competition including song (Wong & Candolin 2005) or aggressive coercion (Casalini *et al.* 2009). Indeed, competition ability is thought to increase with age in many species (Shutler & Weatherhead 1991; Bose & Sarrazin 2007; Laskemoen *et*

al. 2008). However, females can switch partners readily and initial pairings appear to take time (Komdeur 1996), indicating both a "choosing" period and lack of forced social coercion by males. Our data therefore suggest that both age-dependent and, to a lesser extent, heterozygosity-dependent female social mate choice may be operating in these translocated populations, although further experiments would be required to confirm this.

Negative results of mate choice studies are generally unlikely to be published (Bernatchez & Landry 2003; Kotiaho & Puurtinen 2007), but such findings are important in establishing the extent to which active mate choice for functional genes such as the MHC occurs. The results of our study suggest that random social pairing with respect to MHC characteristics occurs in the Seychelles warbler, regardless of whether or not constraints are present. The occurrence of MHC-dependent EPP (Richardson *et al.* 2005) suggests an interaction between MHC genes and fertilisation patterns that is important in maintaining MHC diversity in this species. However, it may be that the historical constraints on, and costs associated with, social mate choice preferences (Kokko *et al.* 2003) have prevented the evolution MHC-dependent social mate choice in the Seychelles warbler, with alternative strategies such as age-dependent choice, promiscuity and male-male competition evolving instead. This study highlights that predicting the occurrence of one sexual selection mechanism, MHC-dependent social mate choice, based on the presence of another, EPP patterns, is not straightforward. There are many potential sexual selection mechanisms that may evolve separately or in concert, and which dominates may differ depending on the constraints that are present (Andersson 1994; Andersson & Simmons 2006). Importantly, understanding how and why particular mechanisms evolve, while others do not, or are observable, while others are not, requires an understanding of the constraints acting upon any given species or population.

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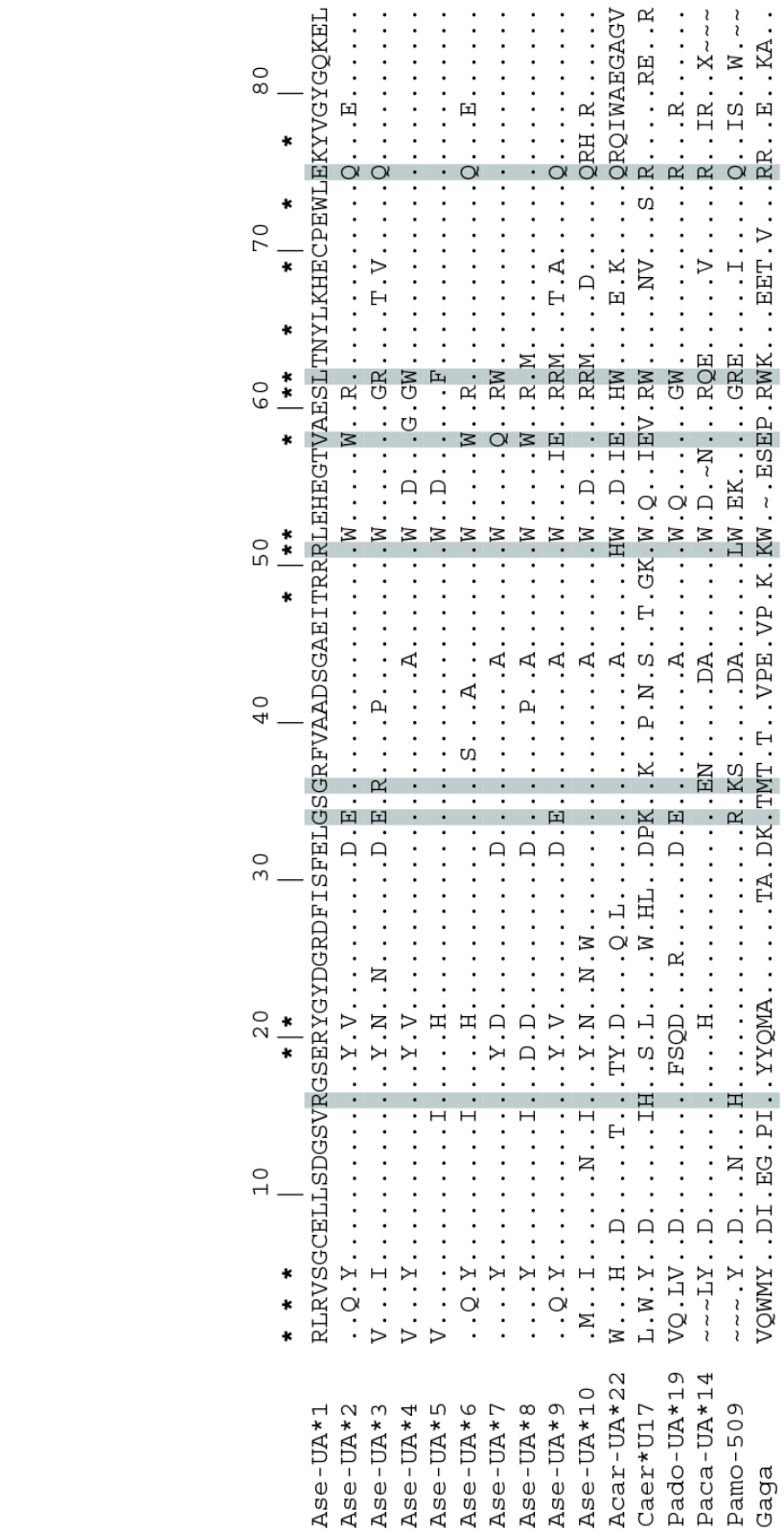
Supplementary Figure S5.1 Amino acid sequences translated from MHC Class I exon 3 cDNA sequences of Seychelles warbler (Ase) with examples of great reed warbler (Acar), scarlet rosefinch (Caer), house sparrow (Pado), blue tit (Paca), green-backed tit (Pamo) and chicken (Gaga, GenBank accession: X12780). Identity with Seychelles warbler is indicated with a dot, gaps with a tilde, sites corresponding to the peptide binding region with an asterisk, and sites identified as positively selected with shaded boxes.



Chapter 6

Investigating MHC-dependent survival and reproduction in the Seychelles warbler

Ever curious…

6.1 Abstract

How evolutionary processes shape adaptive genetic variation and fitness traits is a central question in evolutionary biology. In this chapter, we investigate the dynamics of major histocompatibility complex (MHC) class I variation over time in the Seychelles warbler (*Acrocephalus sechellensis*) population on Cousin. Previous work on a single cohort revealed a five-fold survival advantage conferred by a single allele, *Ase-ua4*. We investigated whether the survival effect is widespread and consistent across the population and whether selection acts to increase the frequency of this allele in the population. We find evidence that *Ase-ua4* increases markedly within each cohort assessed across the temporal span of this long-term study but, surprisingly, that it does not increase in frequency in the population overall. We investigated whether antagonistic effects on survival and reproduction could explain these contrasting results. Using parentage analysis and life history data, we found no difference in the proportion of *Ase-ua4* carriers vs. non-carriers that achieved breeding positions, or how long it took them to do so, although we did find that carriers maintained their breeding position marginally longer. We found no differences in rates of reproductive output, rates of extra-pair and within-pair matings, or the inheritance of *Ase-ua4* in offspring. Further, there was also no clear link between the survival advantage and reproductive senescence as *Ase-ua4* appears to confer its advantage most markedly in mid-life, the period of maximum (female) reproductive output. In conclusion, we find widespread evidence that the reported *Ase-ua4* survival advantage is consistent across time, but no evidence this allele is being driven towards fixation: a result suggestive of an, as yet unidentified, antagonistic effect.

6.2 Introduction

How evolutionary processes shape genetic variation in natural populations is a fundamental question in evolutionary biology. Of particular interest is how selection acts on functional or adaptive variation (e.g. Frankham 1999; Garcia de Leaniz *et al.* 2007) and how this is manifest in key traits such as survival and reproductive success (Ellegren & Sheldon 2008). The relative importance of selection and stochastic processes such as drift in shaping adaptive variation is also of interest (Seddon & Ellegren 2004; Agudo *et al.* 2011), and has conservation implications, particularly in small or bottlenecked populations (Franklin 1980; Frankham *et al.* 1999; Allendorf & Luikart 2007).

The genes of the major histocompatibility complex (MHC) are perhaps one of the best characterised examples to date of the complex interaction between functional variation within the genome and selective pressures from the environment, namely pathogens (Bernatchez & Landry 2003; Sommer 2005; Piertney & Oliver 2006). These hyper-variable genes code for a range of peptide-binding glycoprotein molecules (Hughes & Yeager 1998). Although the MHC forms only one component of the vertebrate immune system (Acevedo-Whitehouse & Cunningham 2006; Ekblom *et al.* 2010), clear relationships between the MHC and survival and reproductive success have nevertheless been documented across diverse taxa (reviewed in Penn 2002; Bernatchez & Landry 2003; Piertney & Oliver 2006). Numerous studies have reported an increase in survival of individuals with either particular MHC genotypes or diversity (e.g. Paterson *et al.* 1998; Brouwer *et al.* 2010; Worley *et al.* 2010; Sepil *et al.* 2012) and, perhaps most remarkably, cases where a single MHC allele influences survival either positively (Pitcher & Neff 2006; Brouwer *et al.* 2010; De Assuncao-Franco *et al.* 2012) or negatively (Carrington *et al.* 1999). Survival and reproduction are closely related fitness traits (e.g. Olsson *et al.* 2005; Dunn *et al.* 2013) and links between the MHC and various reproductive measures such as offspring production and feto-maternal compatibility have also been demonstrated (Kalbe *et al.* 2009; Thoss *et al.* 2011; Aarnink *et al.* 2014), although not ubiquitously (Radwan *et al.* 2012).

The extraordinary diversity observed in the MHC is thought to be maintained by a combination of pathogen-mediated balancing selection and sexual selection (Penn & Potts 1999; Spurgin & Richardson 2010). Three main mechanisms of pathogen-mediated balancing selection are thought to occur. One is heterozygote advantage, where heterozygosity of MHC loci provides more effective antigen recognition compared with homozygous loci (Doherty & Zinkernagel 1975). Another is negative-frequency dependent selection where, due to co-evolution of

parasite and host, rare host alleles provide an advantage in detecting pathogens and subsequently increase in frequency. Once common, they lose their advantage as the parasite evolves counter-measures to avoid detection, but then another rare allele becomes advantageous, creating a cyclical turnover of rare and common alleles, maintaining diversity (Clarke & Kirby 1966). Finally, there is fluctuating selection where spatial and temporal changes in both parasite and host populations create differing selection pressures favouring different MHC alleles and genotypes, thus maintaining diversity over a spatiotemporal scale (Hill 1991). Other mechanisms such as associative balancing complex selection – selection against deleterious mutations in regions linked to MHC genes (Van Oosterhout 2009) – have also been invoked to explain the diversity observed at the MHC.

Disentangling how these numerous mechanisms drive MHC variation is very difficult due to their potential interaction and their similar effects on patterns of MHC variation (Spurgin & Richardson 2010). However, different mechanisms may leave identifiable signatures in allele frequency dynamics over time in a population. For example, negative-frequency dependent selection has been shown to maintain diversity of self-incompatibility loci in plants and can be identified by the rapid increase in frequency of new alleles over time in the population (Wright 1939). Importantly however, this requires medium- to long-term monitoring of populations, which is difficult in vertebrates (Clutton-Brock & Sheldon 2010). It is therefore necessary to employ long-term study systems with adequate temporal sampling to investigate such genetic patterns (Westerdahl *et al.* 2004; Charbonnel & Pemberton 2005). Some studies have also revealed antagonistic relationships between fitness traits such as reproduction and survival, which seem counter-intuitive to evolutionary theory (Kirkwood & Rose 1991; Charmantier *et al.* 2006; Hayward *et al.* 2014). Antagonistic pleiotropy, in which a gene or gene linkage positively affects one trait and negatively affects another (Williams 1957), is one mechanism that can result in an evolutionary trade-off between key phenotypic fitness traits (Charmantier *et al.* 2006; Roff & Fairbairn 2007; Kubinak *et al.* 2012). Such antagonistic effects may hinder our ability to detect the modes of selection maintaining MHC, by disrupting these identifying patterns of allele frequency.

One example of single-allele MHC-dependent survival has been reported in the Seychelles warbler (*Acrocephalus sechellensis*), an insectivorous passerine endemic to the Seychelles archipelago (Safford & Hawkins 2013). Brouwer *et al.* (2010) followed a cohort of individuals for 10 years and, through a comprehensive survival analysis, revealed that a single MHC class I allele, *Ase-ua4*, provided carriers with a five-fold greater life expectancy than non-carriers

(median lifespan of 7.5 vs. 1.5 years respectively). Crucially, this single-allele effect was shown to operate separately to the effects of MHC diversity that influence juvenile survival, and appeared to be consistent throughout the life of the individuals (Brouwer *et al.* 2010). The findings of Brouwer *et al.* (2010) do however pose some questions that remain to be answered. Is the survival effect widespread and consistent through the population over time? Does selection (rare allele advantage or fluctuating selection) act to increase the frequency of *Ase-ua4* over time in the population? Is there interaction between survival and reproductive traits that facilitates the propagation of *Ase-au4* in the population (or indeed, prevents it)?

Here we present an investigation into the dynamics of MHC variation across time in the Seychelles warbler. First we examine the frequency of *Ase-ua4* in the entire Cousin Island population over 19 years (1994–2012). Second, we investigate changes in *Ase-ua4* frequency longitudinally within cohorts and cross-sectionally between age-classes across years (1994–2009). We then examine the reproductive output of MHC-genotyped individuals using a population-wide parentage analysis. We investigate differences in acquisition of breeding status and how this might influence the population-wide frequency of *Ase-ua4.* We investigate inheritance of *Ase-ua4* and compare the productivity *Ase-ua4* carriers vs. non-carriers and the relative proportion of within-pair to extra-pair offspring of social pairs differing in *Ase-ua4* characteristics. Given the strong survival advantage conferred by *Ase-ua4*, we predict that: a) the increase in *Ase-ua4* frequency will be detectable across multiple cohorts over time, b) there will be a significant increase in frequency of the allele within the overall population over time, and c) possessing *Ase-ua4* will result in increased reproductive success as a result of increasing adult survival.

6.3 Materials and methods

6.3.1 Study population sampling

By the mid 20th century the Seychelles warbler was on the verge of extinction due to habitat destruction and the introduction of invasive predators, with the last population of 26–50 individuals (Crook 1960, chapter 2) existing on Cousin Island (4°20'S, 55°40'E, 0.29 km^2). As a result of conservation measures, this population had recovered and stabilised at *ca.* 320 adult individuals, by 1982 (Brouwer *et al.* 2006). The Cousin population has been under intense study since 1986 (>96% individuals ringed since 1997; Richardson *et al.* 2001) as part of a long-term research and conservation project (Komdeur 1992; Richardson *et al.* 2007; Barrett *et al.* 2013). Each year, birds are caught and previously unringed birds are fitted with a unique combination of one metal BTO and three coloured leg rings to allow individual identification. A

ca. 25 µl blood sample is taken from every caught bird and stored in absolute ethanol. All individuals are aged at first catch according to eye-colour and behaviour (Table 6.1) and assigned an estimated hatch date (herein referred to as year of birth, YoB). In most years since 1994 population censuses have been conducted and all reproductive attempts followed during the major (June–September) and (less often) the minor (November–March) breeding peaks (see Komdeur 1991; Richardson *et al.* 2007). Seychelles warblers are strictly territorial, facultative cooperative breeders, with *ca.* 30% of groups comprising not only a dominant pair, but also one or more subordinates that may, or may not, help raise group offspring (Komdeur 1991; Komdeur 1994b; Richardson *et al.* 2007). Each year, based on field observations, every individual is assigned a status according to its social position and breeding activity that year (e.g. dominant breeder, subordinate helper, fledgling etc), and this is entered into the long-term demographic database (Komdeur 1991; Richardson *et al.* 2005). The annual re-sighting probability is 0.98 (± 0.01 SE) for dominant individuals (Brouwer *et al.* 2010) and inter-island dispersal is virtually absent (0.1%, Komdeur *et al.* 2004). We can therefore construct a yearly re-sighting history for each individual and reliably assign the last year they were allocated a status as the estimated death year (Hammers *et al.* 2012), by ensuring that an individual was not recorded for at least two consecutive years after its last known sighting. Where death could not be confirmed (as we were unable to check two consecutive seasons for the last year of the dataset, n = 73), individuals were treated as right-censored where appropriate.

Table 6.1 Age classification system for Seychelles warblers. Eye colour changes, although easily identified, are subject to natural variation and age ranges are approximations based on long-term data (e.g. Komdeur 1991; Hammers *et al.* 2012). Estimated hatch dates are approximate midpoints of age range of age-class.

Age Class	Eye colour	Approximate age range	Behaviour at ringing	Estimated hatch date
Chick	Grey	NA	Still in nest	Ringing date (RD)
Fledgling	Grey	0 – 3 months	Begging	RD – 2 months
Old fledgling	Grey	3 – 5 months	Not begging	RD – 4 months
Sub-adult	Light-brown	5 – 10 months	NA	RD – 8 months
Adult	Red-brown	> 10 months	NA	RD – 1 year

6.3.2 MHC analyses

Variation at exon 3 of class I MHC, which codes for the peptide binding region involved in antigen recognition (Hughes & Yeager 1998), was screened using reference-strand mediated conformation analysis (RSCA) with the primers from Richardson & Westerdahl (2003), following the method of Worley *et al*. (2008). Each segregating RSCA variant corresponded to a

unique 255 bp amino acid coding sequence (hereafter termed "allele" for simplicity, Richardson & Westerdahl 2003). Ten MHC class I alleles have been detected in the Seychelles warbler, with individuals possessing 2–8 alleles each, suggesting that four class I loci are amplified (Richardson & Westerdahl 2003). Our primers were situated within exon 3, and consequently we were not able to screen all the variation within this exon and it is possible that some additional polymorphism exists (e.g. Llaurens *et al.* 2012). However, the amplicon includes all the codons of the peptide-binding region where we expect most variation to be found (Hughes & Yeager 1998). Further, to minimise the effect of this issue we employed two primer sets which vary at the 3' end where a known polymorphisms occurs (Richardson & Westerdahl 2003). Finally, any missed variation would not affect the main results or conclusions of the present study, which investigates changes in frequency of a known allele, linked to survival in this species (Brouwer *et al.* 2010).

6.3.3 MHC allele frequency analyses

All statistical analyses were performed in R v. 2.15 (R Development Core Team 2012) unless otherwise stated. It is impossible at present to identify locus zygosity, due to homogeneity of alleles between multiple, duplicated loci within the passerine MHC (Westerdahl 2007), so frequencies were calculated using presence-absence data. MHC allele frequency was calculated as number of individuals carrying the allele divided by the total number of individuals within the sample. As stochasticity increases with decreasing sample sizes, we limited frequency calculations to samples of ≥4 individuals, to balance this with including as much longevity data as possible. The results are qualitatively the same when changing this limit (e.g. ≥5 individuals, data not shown). We first tested whether carrying *Ase-ua4* was linked to MHC diversity using a bootstrapping approach (Manly 1997). The difference in mean MHC diversity between carriers and non-carriers was calculated (observed difference). We then randomly split the pooled data (1,176 birds) into two groups of equal size to the observed number of *Ase-ua4* carriers and non-carriers (171 and 1,005 respectively) and calculated the difference in mean MHC diversity between them (bootstrapped difference), repeating this pooling and random splitting 10,000 times. The *P* value was then calculated as the proportion of times the bootstrapped difference ≥ observed difference. The population-wide frequency of *Ase-ua4* was calculated for each year 1994–2012 using the re-sighting histories of MHC-typed birds known to be alive during this period. The change in frequency over time was tested using a linear model of allele frequency against years, with logit transformation of the frequency data.

To investigate whether the longitudinal increase in *Ase-ua4* frequency within the single cohort studied by Brouwer *et al.* (2010) was cohort-specific or consistent across time/cohorts, we assessed both longitudinal and cross-sectional allele frequency changes over 19 years. Longitudinal patterns within cohorts were tested by grouping individuals into 3–4 year cohorts by YoB (1994–1996, 1997–1999, 2000–2002, 2003–2005 and 2006–2009) to provide comparable sample sizes to Brouwer *et al.* (2010; Figure 6.2). Only individuals first caught in their YoB (i.e. as chicks, fledglings, or old fledglings, Table 6.1), were included to prevent sampling bias. We excluded winter-hatched (November–March) individuals to standardise age to near-annual intervals corresponding to the main annual fieldwork season (April–October). All individuals translocated to other islands for conservation reasons (n=65; Komdeur 1994a; Richardson *et al.* 2006; chapter 3) were excluded to avoid mistakenly assigning them as dead in cohort analyses (final n = 535). The 1997–1999 cohort used by Brouwer *et al*. (2010) was then excluded to ensure the tests undertaken here were entirely independent of those earlier results. We tested for an increase in *Ase-ua4* frequency within cohorts over time using a linear mixed-effect model with survival years (where: survival year 1 = YoB, the first year of survival) as a continuous, fixed variable and cohort as a random categorical variable, implemented in the LME4 package in R (Bates *et al.* 2014). We used a bootstrapping approach by re-sampling the data with replacement and running the model 2,000 times, from which we took the mean coefficient estimate (β) and 95% confidence intervals. We also modelled the change in frequency of each known MHC class I allele separately for qualitative comparison with *Ase-ua4*. Spearman's rho tests revealed *Ase-ua1* and *Ase-ua10* were completely correlated (ρ = 1.0, Supplementary Table S6.1) so only one of them (*Ase-ua1*) was included in the analyses.

To test for cross-sectional differences in frequency of *Ase-ua4* between different age-classes, we split the dataset according to an individual's chronological age in each year (1994–2009) into; YoB, ≥1 year, ≥5 years or ≥8 years. These classes were selected based on median lifespan (*ca.* 4–5 years, Figure 6.4). We then compared the frequency of *Ase-ua4* between the YoB individuals and each older age-class within each year with linear models of allele frequency, and year and age-class as fixed predictors using one model for each comparison. For this cross-sectional analysis we included both the 1997–1999 cohort and those birds first caught as adults, but excluded winter-born birds as for cohort analyses, to keep annual age intervals standardised (n = 898). Each model was bootstrapped as described for the cohort models and allele frequency data were logit-transformed. We also used Wilcoxon rank-sum tests to confirm the model results.

6.3.4 Parentage assignment

We attempted to assign within-group parentage to 1,506 birds that hatched in the period 1994–2013 and were genotyped at 30 microsatellite loci (Chapter 2). We used maximum likelihood estimation in MASTERBAYES v. 2.451 (Hadfield *et al.* 2006) with Wang's (2004) genotyping error model, following the *MbG_Wang* method of Patrick *et al.* (2012). Allelic dropout and stochastic error rates were both calculated as 0.000 using 47 repeat samples in PEDANT v. 1.0 (Johnson & Haydon 2007), but were set to a conservative default of 0.005. Allele frequencies were taken from genotypes from all years analysed. Year-specific parameters (candidate parents, hatch year and natal territory of offspring) were included to improve assignment probability, which was set at an acceptance threshold of 80% based on marginal probabilities taken from 10,000 iterations. We restricted parentage to the candidate parents in the natal territory to maximise assignment confidence. Where birds were first caught older than five months (age at independence, Table 6.1) and natal territory could not be determined with certainty, we tested parentage using the territory in which the bird was first assigned a status in. We assumed we did not sample all individuals in the population and the number of unsampled males and females were estimated each year. Offspring assigned a mother but no father, were concluded to be a result of extra-pair (group) fertilisations (following Richardson *et al.* 2001; Hadfield *et al.* 2006).

6.3.5 Reproduction analyses

To investigate whether there are any reproductive consequences of carrying *Ase-ua4*, we first tested whether *Ase-ua4* was linked to obtaining a dominant breeding position (i.e. breeding opportunity). We investigated the relative success of carriers vs. non-carriers in obtaining dominant breeding positions across their lifetimes (i.e. from hatching onwards), using the cohort dataset (n = 535) as this contains only individuals caught as chicks, fledglings or old fledglings (Table 6.1). We did this in three ways: 1) using a Fisher's exact test we tested for a difference in the proportion of carriers vs. non-carriers that ever achieved a dominant position; 2) of those that were successful, using a Wilcoxon rank-sum test we tested for a difference in the mean years taken by carriers vs. non-carriers to attain a dominant position; and 3) again using a Wilcoxon rank-sum test we tested the mean years each carrier vs. non-carrier spent as a dominant breeder.

We then compared the annual number of offspring produced by *Ase-ua4* carriers vs. non-carriers. We considered 'offspring' to include all sampled individuals (i.e. from hatching onwards) and assumed missing genotype and offspring data were random with respect to

presence of *Ase-ua4*. The offspring production rate was then calculated as the total number of assigned offspring / the total number of years the individual was known to be alive between 1994 and 2012. The sexes were analysed separately, with the differences assessed using Wilcoxon rank-sum tests. This analysis was based on all MHC-typed birds that were assigned as parents (i.e. not just the cohort dataset). Although socially monogamous, rates of extra-pair paternity (EPP) are high in the Seychelles warbler (40%, Richardson *et al.* 2001), providing opportunity to compare whether *Ase-ua4* carriers differ from non-carriers in proportion of EPP assigned. We analysed the relative proportion of extra-pair paternity lost to pair males where either: both, one or neither of the social pair carried *Ase-ua4*. This test was blind to offspring MHC genotype. In instances of extra-pair paternity, the dominant breeding male recorded in that territory at that time was assumed to be the social pair male of the mother. As paternity was only assigned to within-pair (group) males, it was not possible to compare the MHC genotype of the cuckolding male. We tested for a difference in observed vs. expected proportions of within-pair and extra-pair offspring assigned to each category of social pair using Fisher's exact test.

To visualise the stage at which carrying *Ase-ua4* influences survival relative to reproductive output, we constructed Kaplan-Meier survival curves for carriers vs. non-carriers of *Ase-ua4* using the cohort data (n=535) in the R package SURVIVAL (Therneau 2014). A total of 73 individuals were alive at the end of the study period and were right-censored. We then overlaid data on female reproductive senescence from Hammers *et al.* (2012) to qualitatively assess how *Ase-ua4* conferred survival advantage overlaps with the age-related distribution of reproductive senescence.

Finally, we tested whether the inheritance of *Ase-ua4* adhered to Mendelian expectations. We took families in which the offspring and both parents were MHC-typed and grouped them by parental *Ase-ua4* genotype (i.e. both, one or neither parent carrying *Ase-ua4*). We determined the observed frequency of *Ase-ua4* allele inheritance in each category (Table 6.2). We then calculated the expected number of offspring carrying *Ase-ua4* for each parental category. We assumed *Ase-ua4* to be an allele at a single locus and (conservatively) parents with the *Ase-ua4* to be carrying only one copy of that allele (i.e. heterozygous) at that locus, although we note zygosity is unknown in this species and acknowledge the limitations these assumptions. We tested observed vs. expected number of offspring carrying *Ase-ua4* using a χ^2 test.

6.4 Results

Status histories and MHC-genotypes were compiled for 1,176 individuals. Microsatellite genotypes were compiled for 1,704 individuals with 98.6% completion. Parentage was determined for 984 offspring: both parents in 613 cases, mother only in 371 cases (thus identified as extra-pair paternity). Parentage could not be assigned in 522 cases because 1) parent-offspring assignments with <80% probability were excluded, and 2) many individuals were first caught in non-natal territories where none of the candidates was a true parent.

6.4.1 MHC allele frequencies

There was no difference in mean MHC diversity between *Ase-ua4* carriers and non-carriers (bootstrap test P = 0.26). The mean frequency of *Ase-ua4* in the Cousin Island population remained constant at *ca.* 0.15, with no overall change over time (P = 0.83, Figure 6.1A). In striking contrast, there was a significant longitudinal increase in the frequency of *Ase-au4* with age in all four cohorts assessed (survival years β = 0.11, 95% CIs = 0.08–0.14, Figure 6.1B). Furthermore, *Ase-ua4* was the only allele of the 10 known MHC class I alleles to show a significant positive frequency increase in this way (Figure 6.2, Supplementary Figure S6.1).

When allele frequency was measured by chronological age-class, there was no difference in frequency of *Ase-ua4* between YoBs vs. ≥1 year (i.e. in their second year of life) old birds (P = 0.83), a marginal difference between YoBs vs. ≥5 year old birds (P = 0.05) and a significant difference between YoBs vs. ≥8 year old birds across years (P = 0.01, Figure 6.3, Supplementary Figure S6.2). Wilcoxon rank-sum tests confirmed the same pattern of frequency differences over time as the models (YoB vs. ≥1 year, W = 91.5, P = 0.17; YoB vs. ≥5 years, W = 69, P = 0.03; YoB vs. ≥8 years, W = 53, P = 0.005).

6.4.2 Ase-ua4 and reproduction

There was no difference in the proportion of *Ase-ua4* carriers vs. non-carriers achieving a dominant breeding position (N given with [proportion]: 'carriers', dominant status = 39 [0.55] vs. no dominant status = 32 [0.45]; 'non-carriers', dominant status = 222 [0.49] vs. no dominant status = 242 [0.51], P = 0.31). Of those that did gain a dominant position there was no difference in the amount of time (years) taken to gain that position (mean ± SD, carriers = 2.97 ± 0.74, non-carriers = 3.08 ± 0.95, W = 4202.5, P = 0.75). No sex differences were detected for any test (all P > 0.06). However, there was a marginal difference in the time spent as a breeder, with *Ase-ua4* carriers maintaining the position longer than non-carriers (W = 5254, P = 0.032; Supplementary Figure S6.3).

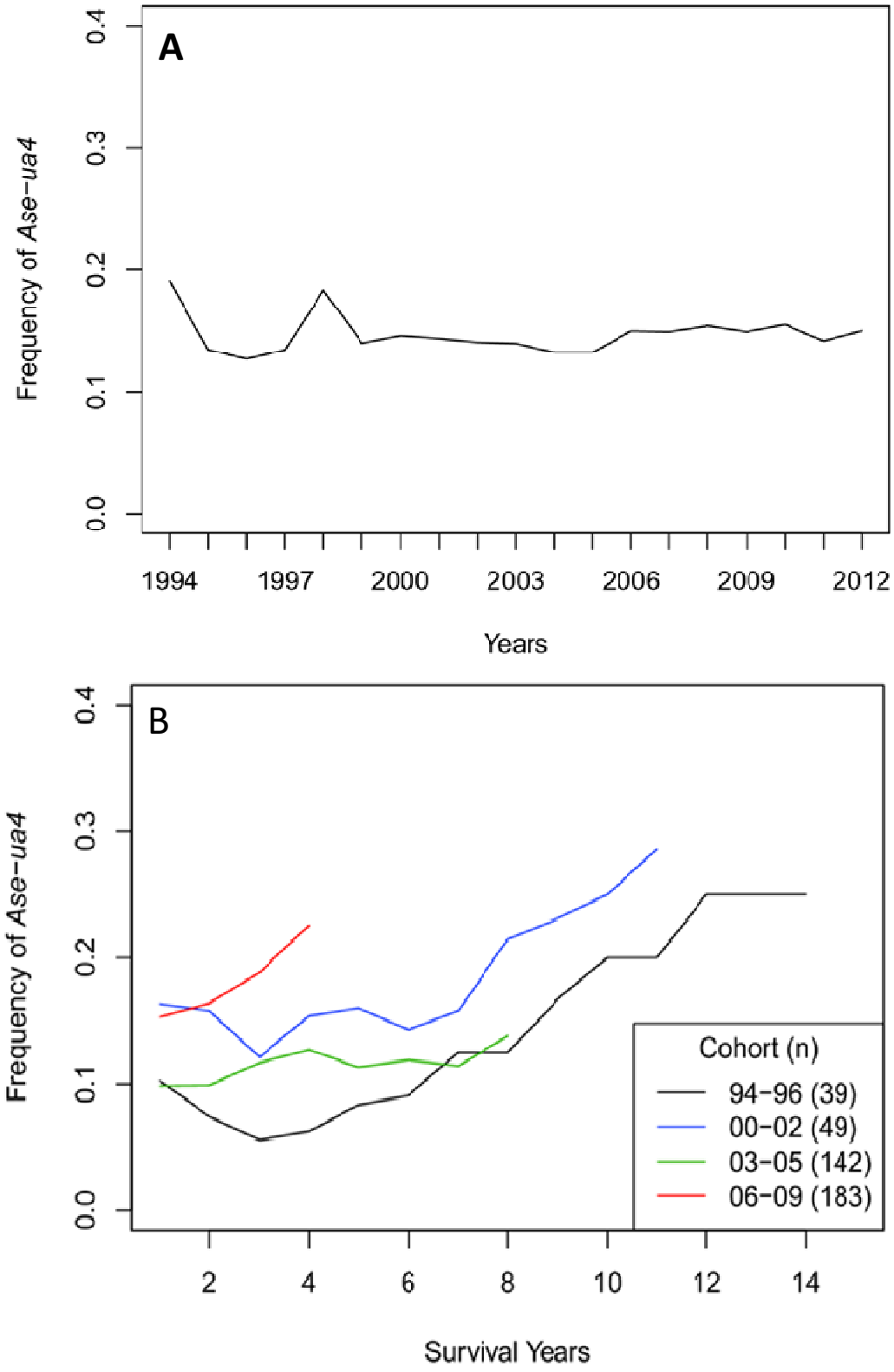


Figure 6.1 A) Frequency of MHC class I allele *Ase-ua4* over 19 years in the Cousin Island population of Seychelles warblers and **B)** Longitudinal frequency of *Ase-ua4* across four cohorts of Seychelles warblers followed on Cousin Island from 1994 to 2012. X-axis survival year 1 corresponds to first year of survival i.e. year of birth (YoB).

No difference was found in the mean rate of offspring produced between *Ase-ua4* carrier and non-carrier females (mean ± SD; carriers = 0.47 ± 0.32, non-carriers = 0.46 ± 0.27, W = 5478, P = 0.85) or males (carriers = 0.36 ± 0.21, non-carriers = 0.39 ± 0.23, W = 2855.5, P = 0.71). There was also no difference in the proportion of within-pair to extra-pair offspring produced by social pairs that differed in whether or not they carried the *Ase-ua4* allele (N given with [proportion]: 'both carriers of *Ase-ua4*', WP = 22 [0.67] vs. EP = 11 [0.33]; 'one carrier', WP = 109 [0.62] vs. EP = 66 [0.38]; 'neither carriers', WP = 331 [0.68] vs. 155 [0.32], Fisher's exact test P = 0.37). The Kaplan-Meier curves suggested that the survival advantage of *Ase-ua4* was most prominent in mid-life (Figure 6.4) when (at least female) productivity is highest (Hammers *et al.* 2012). There was no difference in observed and expected inheritance of *Ase-ua4* by within-pair offspring of each *Ase-ua4* category of parent (where: both, one or neither are carriers), assuming a single locus allele and heterozygous parents (χ^2 = 1.752, P > 0.1, Table 6.2).

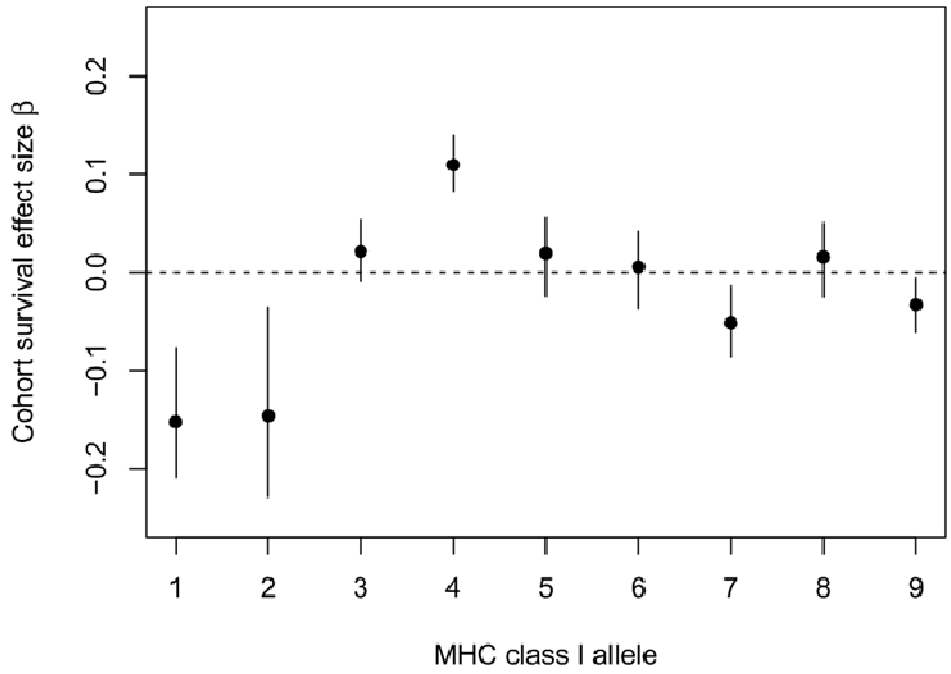


Figure 6.2 Coefficient (β) estimates with 95% confidence intervals for linear mixed-effect models of MHC class I allele frequency with survival years as a fixed variable and cohort as a random variable. Each allele is modelled separately. *Ase-ua1* and *Aseu-a10* are correlated (ρ=1.0) so only *Ase-ua1* was analysed. Mean bootstrapped coefficients and confidence intervals are presented.

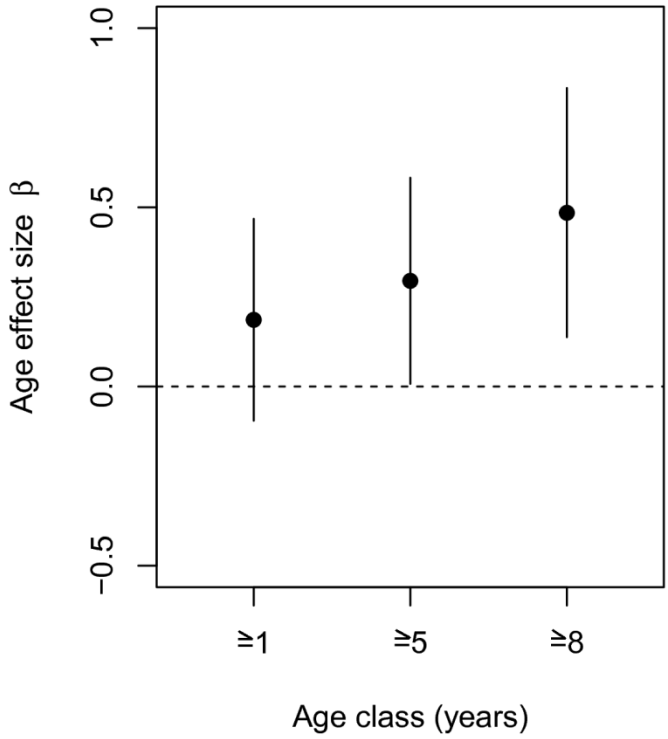


Figure 6.3 Mean bootstrapped coefficient (β) estimates with 95% confidence intervals for linear models of MHC class I allele *Ase-ua4* frequency comparing YoBs (birds born in that year) with different age classes in each year from 1994 to 2009.

Table 6.2 Inheritance pattern of *Ase-ua4* in the Seychelles warbler population on Cousin Island. Family = 1 offspring + mother + father, where all birds are MHC-typed. Expected values calculated assuming single locus and heterozygote parents.

Families (n)	Parental *Ase-ua4*	Offspring *Ase-ua4*	
		N expected (%)	N observed (%)
12	Both carriers	9 (75)	8 (67)
78	One carrier	39 (50)	31 (40)
245	Neither carriers	0 (0)	0 (0)

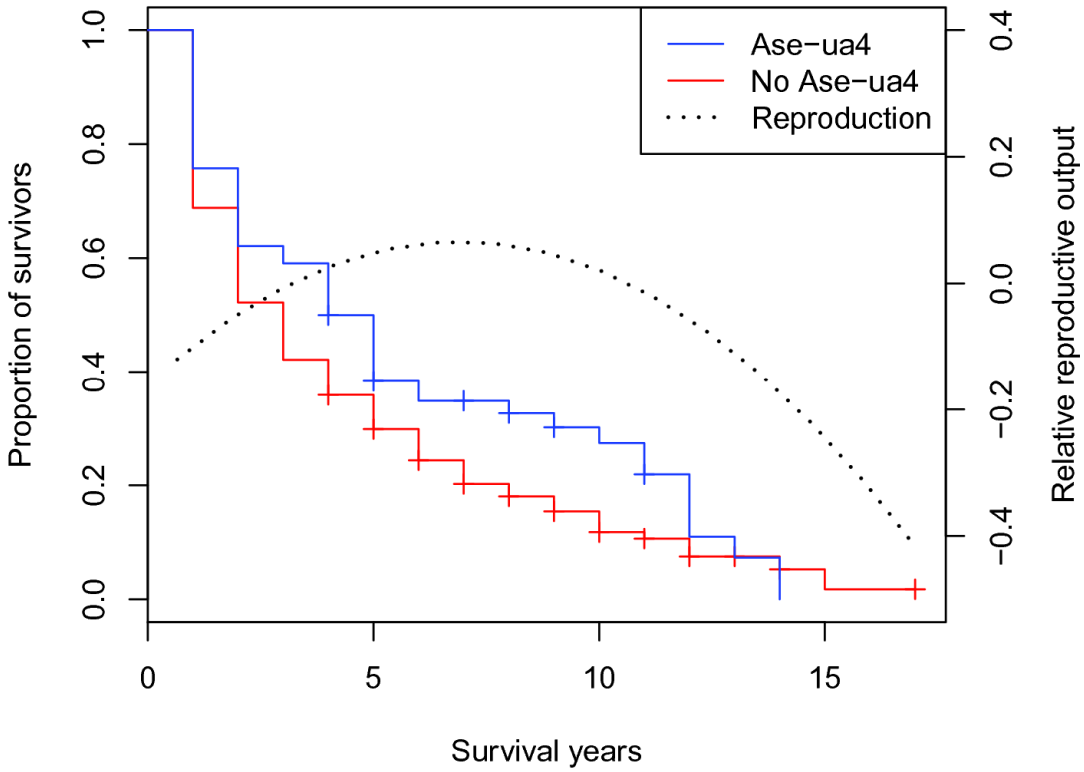


Figure 6.4 Kaplan-Meier survival curves of carriers and non-carriers of *Ase-ua4*. The dashed line shows female reproductive senescence (z-axis). Relative reproductive output is the number of fledglings produced by a female minus the averaged reproduction of all females in a given year. The curve shows the peak and subsequent decline of reproductive output according to female age. *Senescence data reproduced unmodified from Hammers et al. (2012) with permission.*

6.5 Discussion

In this chapter, we investigated the evolutionary mechanisms influencing the long-term frequency of *Ase-ua4*, a class I MHC allele that has been shown to have a strong survival advantage (Brouwer *et al.* 2010). We found evidence of increasing frequency of the allele both within cohorts as they age and across age-classes in the population. However, in striking contrast there was no increase in frequency of *Ase-ua4* over time in the population overall. We hypothesised that this could be because of an antagonistic effect of *Ase-ua4* reducing the relative levels of reproductive success despite increasing survival. However, we detected no effect of *Ase-ua4* on an individual's ability to gain a dominant breeding position, but carriers did hold their dominant breeding position marginally longer than non-carriers. Similarly, no effects of carrying *Ase-ua4* on reproductive output or relative proportions of within- vs. extra-pair offspring were detected. We showed that actually the survival advantage conferred by *Ase-ua4* was most prominent during the period of maximum (female) reproductive output. We

also found no evidence to suggest that the inheritance of *Ase-ua4* deviated from Mendelian expectations, assuming a single-locus allele and two heterozygous parents.

Based on the work of Brouwer *et al.* (2010) we predicted that there would be a significant increase in frequency of *Ase-ua4* over time in the Seychelles warbler population on Cousin due to the significant survival advantage this allele confers on its carriers. The remarkable stability of *Ase-ua4* at a frequency of *ca.* 0.15 in the population (Figure 6.1A) over 19 years is therefore a surprising result. One potential explanation for this is that the survival effect demonstrated by Brouwer *et al.* (2010) was cohort-specific. Cohort effects can significantly influence life history traits (Rose *et al.* 1998; Lindstrom & Kokko 2002; Hastings *et al.* 2011). Using the data from the present study, this possibility can now be refuted. We found a consistent increase in frequency of *Ase-ua4* over time within four additional cohorts spanning 19 years overall. As expected in a natural, unmanaged population (Lindstrom & Kokko 2002), the rate of *Ase-ua4* frequency increase appears to vary between cohorts (Figure 6.1B) but it is nonetheless consistently positive. Importantly, our analyses did not include the birds within the cohort assessed by Brouwer *et al.* (2010) and so independently confirmed the importance of this allele in the population over time.

Ase-ua4 was the only allele (out of 10 studied) to show a significant positive effect on survival in the comprehensive survival analyses undertaken by Brouwer *et al.* (2010) and, accordingly, it is the only allele to show significant positive frequency increase in the current analyses (Figure 6.2). The majority of the other alleles were either neutrally or marginally negatively associated with number of years survived within a cohort. *Ase-ua1* and *Ase-ua2* both show a more significant negative relationship (larger β estimates), but the confidence intervals around these estimates suggest a much higher degree of uncertainty in their true effect sizes, and previous work found no evidence to suggest they are linked to survival in any way (Brouwer *et al.* 2010). We also detected a cross-sectional pattern of frequency differences, where individuals born in any given year (YoBs) have marginally lower *Ase-ua4* frequency than ≥5 year old individuals and significantly lower than ≥8 year old individuals (Figure 6.3), a pattern corroborated by two separate statistical approaches. This pattern further supports the findings of Brouwer *et al.* (2010) that individuals carrying *Ase-ua4* survive longer than those that do not and indicates that *Ase-ua4* allele-specific survival is actually widespread over time within the population.

Few studies have investigated long-term changes in MHC allele frequency that span multiple generations in natural populations. A study by Westerdahl *et al.* (2004) on great reed warblers (*Acrocephalus arundinaceus*) reported that fluctuations in two alleles exceeded random expectation, and concluded that fluctuating selection was responsible. A similar study by Charbonnel & Pemberton (2005) found evidence of fluctuating selection in the MHC of Soay sheep (*Ovis aries*), whereas one study on wolves (*Canis lupus*) found evidence of temporal MHC allele changes consistent with neutral evolution (Seddon & Ellegren 2004). If negative frequency-dependent selection was acting in the Seychelles warbler, we would not expect the population level frequency of *Ase-ua4* to remain stable of over time, while if fluctuating selection were acting, we would not expect consistent patterns of *Ase-ua4* frequency increase within cohorts over time, or a stable population-wide allele frequency. Consequently this study does not provide any evidence of either fluctuating or negative frequency-dependent selection acting on *Ase-ua4* at least over the generations of Seychelles warbler that we observed. To our knowledge, no other published studies have followed the change in frequency of a single MHC allele associated with a known survival effect over multiple generations within a population, so we are limited in comparisons with the wider literature. More and even longer-term studies are needed before we can determine what the patterns we have observed can really tell us about the mechanisms that drive variation in the MHC in wild populations.

A five-fold survival advantage conferred by an allele would inevitably cause an increase the frequency of that allele within a population over time, all else being equal. That the survival effect is consistent in the population over time, but does not result in a population-wide increase in *Ase-ua4* frequency, indicates that the allele is failing to propagate as expected. This suggests there may be an opposing selective pressure, such as a reproductive cost associated with *Ase-ua4*. One possible explanation for this is antagonistic pleiotropy (Williams 1957; Rose 1982). It may be that, in the Seychelles warbler, carrying *Ase-ua4*, or an allele at a closely linked gene, provides survival benefits at the cost of lower reproductive success. Such a mechanism could lower an individual's reproductive ability or ability to obtain and maintain a dominant breeding position. A second, closely related, explanation could be the expression of a sheltered mutational load when carriers of *Ase-ua4* mate, following the ABC hypothesis (Van Oosterhout 2009). Van Oosterhout (2009) postulated that the MHC experiences reduced recombination, functioning in 'epistatic blocks'. This allows a 'Muller's ratchet'-type accumulation of deleterious mutation (Muller 1932), which is effectively sheltered from purging by strong selection acting upon the MHC genes (Van Oosterhout 2009). This sheltered load then adds to balancing selection on MHC by reducing the co-inheritance of the same

'epistatic blocks', thus maintaining diversity. Seychelles warbler offspring viability may be adversely affected by the expression of deleterious mutations linked to *Ase-ua4* when matings between carriers occurs, lowering the overall reproductive success of carriers compared with non-carriers. Under this scenario, we would expect a greater relative proportion of offspring of social pairs comprising two carriers to result from extra-pair copulations compared with social pairs comprising one or no carriers. This is because, given that 40% of all offspring are extra-pair (Richardson *et al.* 2001), we might expect fewer within-pair offspring from carrier pairs to survive and thus a larger proportion of carrier-pair offspring to consist of extra-pair young. Although both these hypothesised mechanisms (antagonistic pleiotropy and ABC) are very similar and would provide identical outcomes for allele frequencies, they differ in that pleiotropic genes provide both positive and negative effects either in certain circumstances, or at certain life stages, whereas in the sheltered load mechanism mutations provide only negative consequences, and then only when expressed through homozygosity.

The parentage analysis presented in this chapter enabled us to screen for evidence that the mechanisms outlined above were acting on reproductive success in some way to maintain *Ase-ua4* at a constant, low frequency. Interestingly, we failed to detect any evidence for either antagonistic pleiotropy or ABC. *Ase-ua4* carriers and non-carriers were equally capable of attaining a breeding position, equally quickly. The slight difference in length of time carriers held breeding positions compared with non-carriers is expected if carriers are generally living longer. Moreover this pattern would reinforce the benefits of being an *Ase-ua4* carrier rather than indicate an antagonistic effect. For that one would need to find that *Ase-ua4* carriers were less able to gain or hold a dominant breeding position. Furthermore, the rate of offspring production was similar for both carriers and non-carriers and the relative proportions of within-pair to extra-pair offspring were no different between pairs of parental birds with different combinations of the *Ase-ua4* allele. Furthermore, although our analyses are on a relatively limited sample (due to the low frequency of *Ase-ua4*) the inheritance of this allele at least provides no evidence to suggest any inhibition of *Ase-ua4* inheritance from carriers to offspring. Although we must be careful in interpreting lack of evidence as explanation, assuming two heterozygote parents provides a conservative estimate of the expected allele frequencies in offspring which matches our observed data.

Finally the Kaplan-Meier survival curves suggest that *Ase-ua4* survival benefits are most pronounced in mid-life (Figure 6.4). This is the same period of time that female Seychelles warblers experience their greatest relative reproductive contribution to the population, as

seen from the senescence curve data from Hammers *et al.* (2012; Figure 6.4). Although comparable data for males are not currently available, we expect that males would show a similar pattern, with perhaps less or later reproductive senescence than in females, as observed in other species (Vom Saal & Finch 1988; but see: Nussey *et al.* 2009; Dugdale *et al.* 2011). Collectively, the tests outlined above were unable to detect any mechanism that signifies that carriers of *Ase-ua4* may suffer lower levels of reproductive success antagonistic to the increased survival benefits gained. In fact, based on our evidence, *Ase-ua4* carriers should be achieving higher reproductive output overall as they are equally adept at reproduction as non-carriers and both survive and maintain dominant breeding positions for longer.

Are there other potential explanations that may explain our results? It is possible that we did not detect antagonistic effects because our sampling was biased or too limited. Although all breeding attempts are followed during the peak breeding seasons (Richardson *et al.* 2007), a small number of territories may attempt breeding at any time of year (Komdeur 1991; Komdeur 1996) and may therefore be unrecorded. A number of offspring are also not sampled because of egg or chick predation (Komdeur & Kats 1999) and some nests high in trees cannot be accessed by fieldworkers. Furthermore, the parentage analysis was restricted to candidate parents known to be present in a territory in a given breeding season. A number of offspring were not successfully assigned parentage due to assignment failure or low probability. This is probably due to parents existing outside the breed groups (i.e. offspring sampled outside their natal territory and thus of unknown origin). Our analyses will therefore have marginally underestimated the true number of offspring produced, but we assumed that the extrinsic factors affecting nest success (such as weather events or predation) and sampling effort were random with respect to parental and offspring *Ase-ua4*. However, we were unable to quantify or assign failure of breeding attempts to either extrinsic factors such as predation or intrinsic factors such as infertile eggs that may be linked to *Ase-ua4*. It is therefore possible that our methods and sampling window (19 years) were not sensitive enough to detect a subtle effect.

Our data suggest that, although the *Ase-ua4* effect appears to be widespread in the population, there does not seem to be a clear reproductive mechanism preventing it from increasing in frequency in the population. One consideration is the strength of selection for the survival advantage and whether this is sufficient to increase the frequency of the *Ase-ua4* in the population. The Cousin island population is small at *ca.* 320 ringed individuals (Brouwer *et al.* 2007). Many studies show that in small populations drift can overpower selection as the

dominant force shaping adaptive variation (e.g. Kimura 1983; Seddon & Ellegren 2004; Miller *et al.* 2010; Agudo *et al.* 2011; Chapter 3). Furthermore, when effective population sizes are small, even alleles with strong favourable effects can behave neutrally ($s \leq 1/2N_e$, Whitlock 2000; Ferrière *et al.* 2004). The effective population size of the Seychelles warbler population on Cousin Island is between *ca.* 30 and 80 (Chapters 2 & 3). This raises the possibility that even though *Ase-ua4* confers a strong phenotypic effect, it is simply not strong enough to overcome stochastic effects inherent in such a small population, resulting in the failure of the allele to propagate as predicted under a scenario in which selection dominated.

The power of long-term studies such as the Seychelles warbler system lies in our ability to identify and investigate long-term, population- and individual-level effects (e.g. Charbonnel & Pemberton 2005; Nussey *et al.* 2009; Clutton-Brock & Sheldon 2010). Even though the mechanism preventing the propagation of *Ase-ua4* in the population remains unclear, we have been able to show a consistent and intriguing effect across the population and highlight how predictions regarding the behaviour of genetic variation in populations may not concur with reality. This study also provides an interesting example of combining longitudinal and cross-sectional analyses with parentage data in uncovering population- and individual-level genetic patterns in a natural population.

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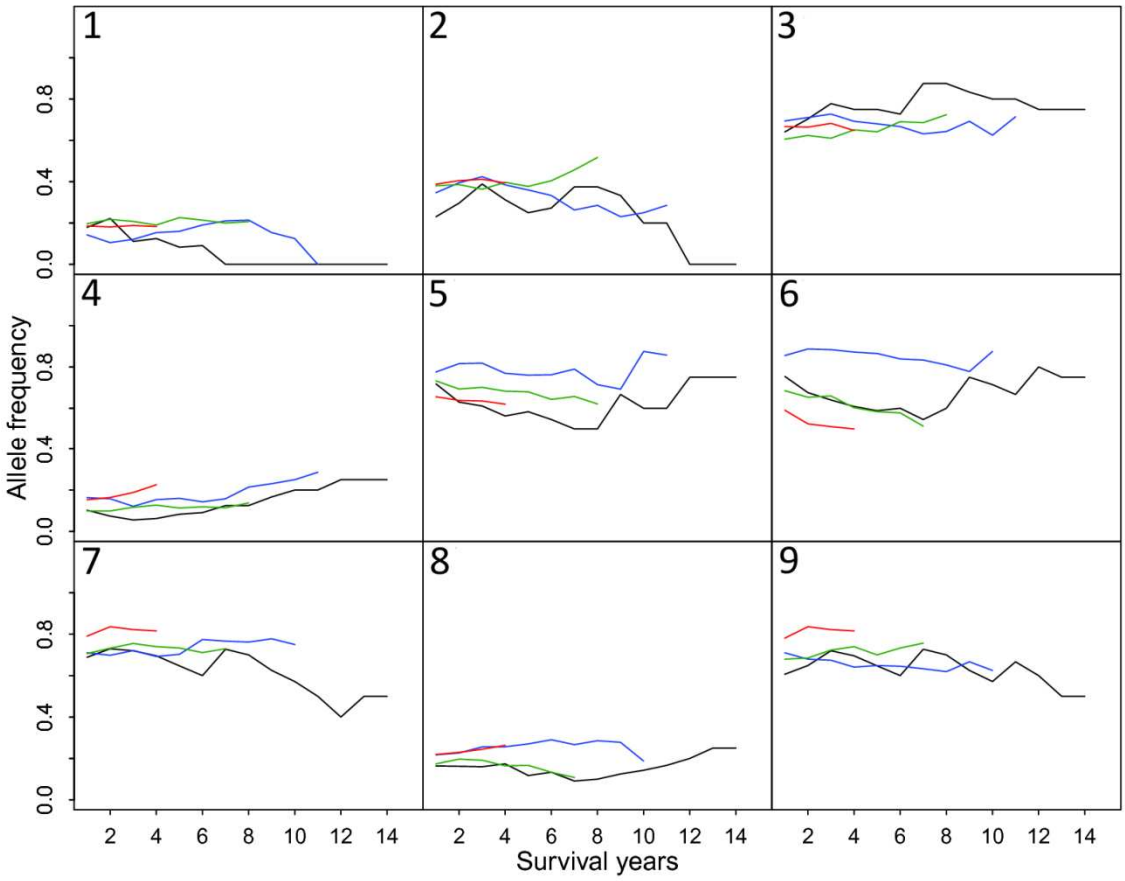
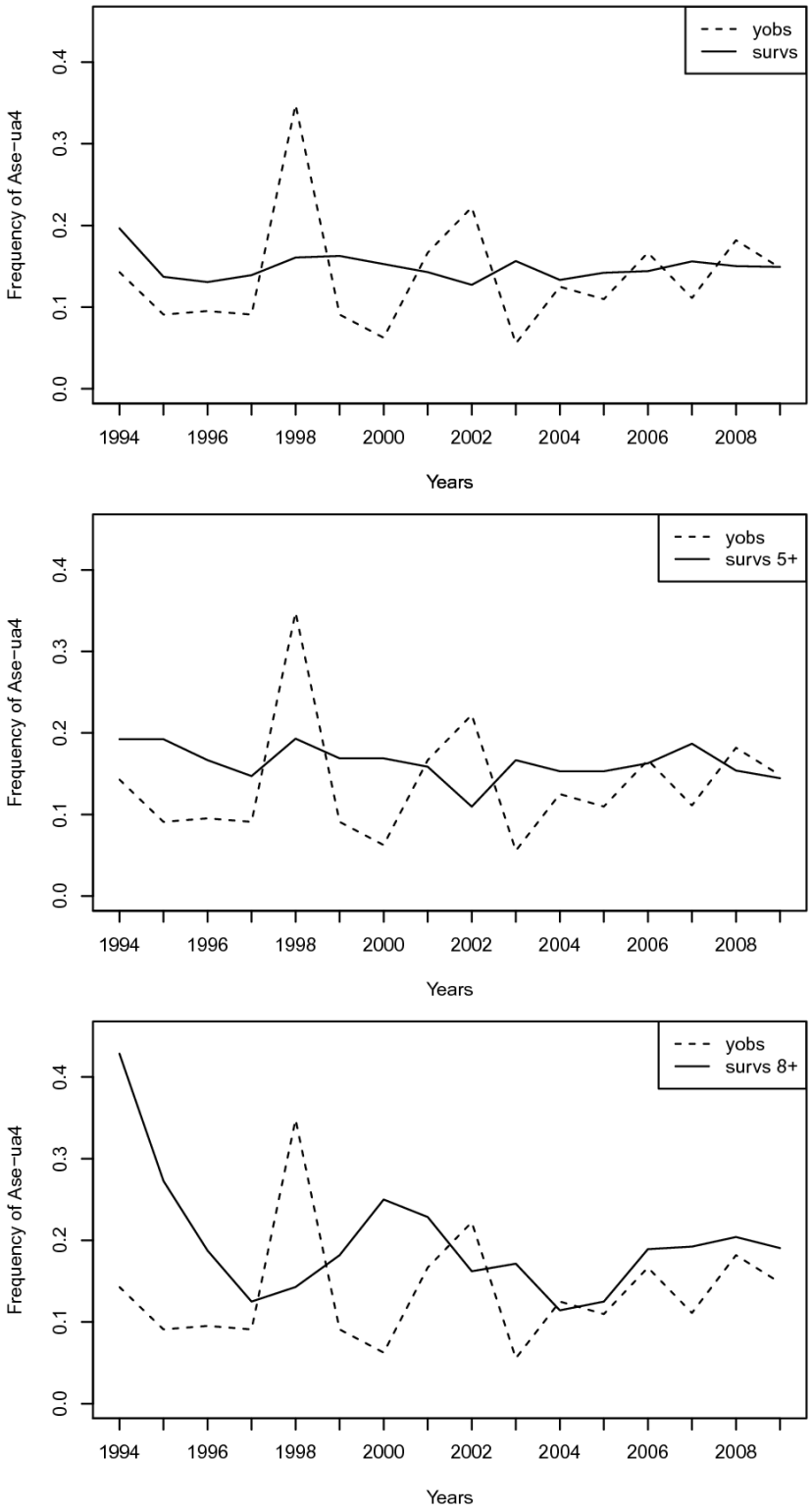
Supplementary Table S6.1 Spearman's rho correlations between 10 MHC class I alleles in the Cousin population of Seychelles warblers. Rho given in lower matrix and *P* values given in upper matrix.

	Aseua1	*Aseua2*	*Aseua3*	*Aseua4*	*Aseua5*	*Aseua6*	*Aseua7*	*Aseua8*	*Aseua9*	*Aseua10*
Aseua1		0.000	0.013	0.001	0.000	0.000	0.000	0.000	0.000	0.000
Aseua2	-0.207		0.000	0.120	0.000	0.000	0.000	0.000	0.000	0.000
Aseua3	-0.108	0.472		0.001	0.000	0.000	0.000	0.028	0.000	0.013
Aseua4	-0.139	-0.067	-0.141		0.004	0.001	0.000	0.091	0.001	0.001
Aseua5	-0.262	-0.188	-0.434	-0.124		0.000	0.000	0.000	0.000	0.000
Aseua6	-0.262	-0.222	-0.417	-0.148	0.868		0.000	0.000	0.000	0.000
Aseua7	0.297	0.403	0.763	-0.179	-0.368	-0.368		0.000	0.000	0.000
Aseua8	0.521	-0.212	0.095	-0.073	-0.294	-0.244	0.301		0.154	0.000
Aseua9	-0.158	0.470	0.822	0.141	-0.396	-0.387	0.670	0.062		0.000
Aseua10	1.000	-0.207	-0.108	-0.139	-0.262	-0.262	0.297	0.521	-0.158	

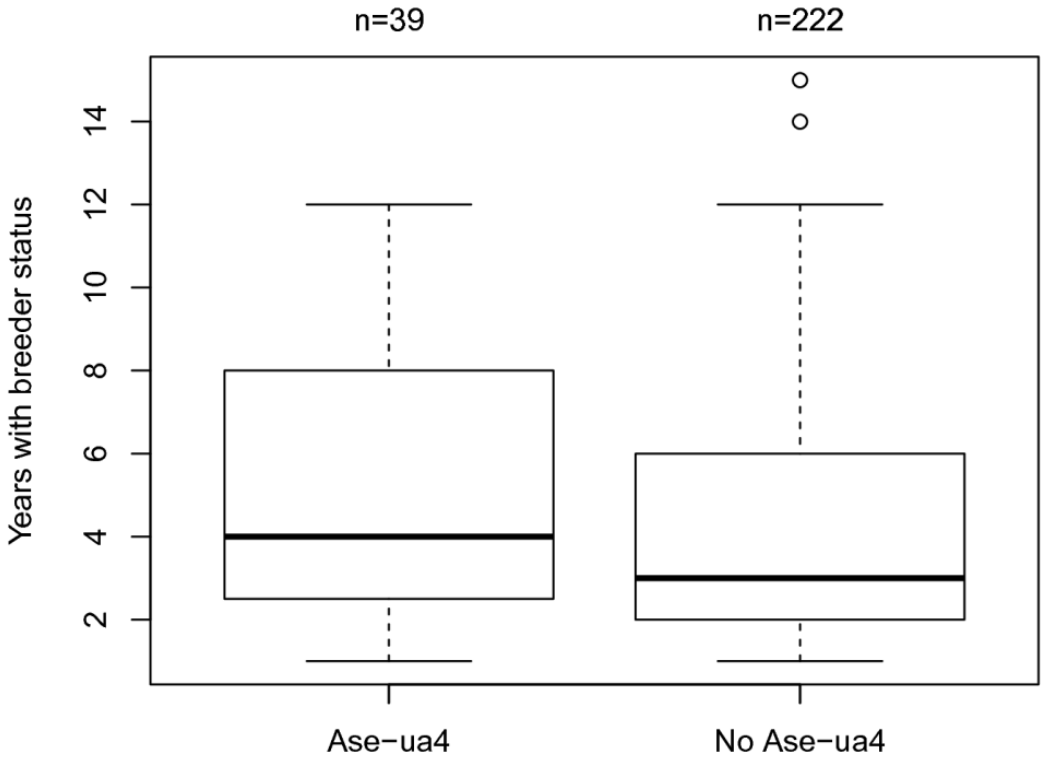
Supplementary Figure S6.1 Cohort allele frequencies across 9 class I MHC alleles (*Ase-ua1* & *Ase-ua10,* ρ = 1, *Ase-ua10* not shown). Cohorts are; black 1994–1996, blue 2000–2002, green 2003–2005 and red 2006–2009. Alleles *Ase-ua1-9* displayed 1-9 respectively.



Supplementary Figure S6.2 The frequency of MHC Class I allele *Ase-ua4* in different age-classes of Seychelles warblers on Cousin Island. YoBs – individuals first caught in hatch year as chicks, fledglings or old fledglings; Survs – individuals ≥1 year old; Surv 5+ is individuals ≥5 years old; Surv 8+ is individuals ≥8 years old in each year 1994–2009.



Supplementary Figure S6.3 Boxplot showing the differences in the duration that carriers and non-carriers spend in a socially dominant breeding position. Carriers spend marginally longer as breeders than non-carriers, *P* = 0.032.



Chapter 7

General discussion

A Seychelles sunset viewed from the research station on Cousin Island

7.1 General discussion

Throughout this thesis, I have emphasised the emergence of evolutionary conservation as a discipline and the shift towards a more thorough integration of conservation and evolutionary concepts (Crandall *et al.* 2000; Ferrière *et al.* 2004; Allendorf & Luikart 2007; Carroll & Fox 2008; Höglund 2009). The purpose of this thesis was to investigate how evolutionary processes and conservation action interact to shape genetic variation in natural populations. Specifically, I aimed to investigate how these factors differentially influenced neutral and adaptive variation. I used microsatellites for quantifying neutral variation and the MHC as candidate genes for adaptive variation, and studied natural populations of the Seychelles warbler, an ideal model species for exploring the evolutionary conservation approach. Here, I discuss my findings in a collective context and outline avenues for further research.

7.1.1 An evolutionary conservation case study

A key aspect of evolutionary conservation is gaining understanding of the evolutionary history of a species, its impact on contemporary population- and individual-level characteristics and how this might influence conservation measures. In Chapter 2, I provided evolutionary context to the genetic variation observed in the contemporary population of Seychelles warblers. Using museum DNA, I was able to show a recent (120-250 years ago) and severe decline in population size and a 25% loss of microsatellite diversity, which both confirmed and quantified the severity of the bottleneck that had been documented over the last 150 years in this species (Oustalet 1878; Vesey-Fitzgerald 1940; Crook 1960; Collar & Stuart 1985). The effective population size estimates I produced provide evidence that the Seychelles warbler had a widespread historical distribution probably spanning at least the inner granitic islands of the Seychelles (see Figure 1.1, Chapter 1) with gene flow occurring between them. This is because a population's effective size is generally around a tenth of its census size (Allendorf & Luikart 2007; Frankham *et al.* 2010), which infers the Seychelles warbler must have once existed in tens of thousands. This has several important implications. Given the short timescale since the bottleneck, purging is unlikely to have removed the genetic load from the population (Crnokrak & Barrett 2002) and, in accordance with this, we know that inbreeding depression does still affect Seychelles warbler fitness (Richardson *et al.* 2004). It may also provide some explanation for why we observe both high levels of extra-pair paternity (Richardson *et al.* 2001) and a lack of inbreeding avoidance (Richardson *et al.* 2004; Eikenaar *et al.* 2008). Both of these are atypical attributes for an island species but expected in larger populations (Griffith 2000; Jamieson 2011). It may simply be that we are observing a time-lag effect, with the contemporary population displaying characteristics of a much larger historical population (Blumstein 2002).

I had intended to screen for historical MHC diversity in the museum samples to provide comparative estimates of loss of adaptive variation. Unfortunately, this was not possible owing to the low quantity and degraded nature of the museum sample DNA. The MHC exon 3, at 274 bp (Richardson & Westerdahl 2003), was at the upper limit of the microsatellite fragment sizes we were able to genotype reliably (Chapter 2). I considered designing shorter, overlapping primer sets to amplify the MHC in sections. However, given the distribution of variation in the exon sequence (and assuming even greater variability in the museum samples) designing fragments of satisfactory length without the need to amplify each museum sample an excessive number of times was not feasible. Considering the high error rates and need for repeat genotyping in museum DNA (Wandeler *et al.* 2007; Arandjelovic *et al.* 2009), there was insufficient DNA sample to run adequate error checks and we could not justify exhausting the sample we had for unreliable data.

Acknowledging the evidence for a recent and severe loss of diversity presented in Chapter 2, with its implications for inbreeding depression (Richardson *et al.* 2004) and evolutionary potential, the next consideration was the genetic fate of the translocated populations. Translocations typically cause bottlenecks and founder effects by incomplete sampling of the source population that occurs with small founder sizes (Stockwell *et al.* 1996; Jamieson 2011; Weiser *et al.* 2013). In Chapter 3, I found that the earlier translocations of small numbers of founders (n = 29) to Aride and Cousine (in 1988 and 1991) has resulted in moderate but significant genetic divergence (F_{ST} = 0.08) between these populations, suggesting conservation concerns were well founded. In terms of absolute diversity I showed that, contrary to our expectations, there was actually little further loss of diversity overall in the translocated populations, but that it was the arrangement of this variation in terms of allele frequencies that was significantly affected. That little diversity was lost as a result of translocation may be a direct result of the historical bottleneck, as this already meant that there was little remaining diversity to be lost. These effects in the Seychelles warbler mirror effects seen in other studies such as that on the New Zealand saddlebacks (*Philesturnus carunculatus*; Taylor & Jamieson 2008). However, as we had complete sampling of founders in two of the four translocations, I was able to show that loss of individual microsatellite alleles did occur, mostly from the more diverse loci, thus highlighting that even in genetically depauperate populations, we still have incomplete sampling issues with small translocation sizes (Stockwell *et al.* 1996; Hedrick 1999). The diversity across the multiple Seychelles warbler populations was temporally stable, suggesting drift has had minimal effect in further eroding diversity in the translocated populations. In conservation terms, this is very positive news as it means that the populations grew, or are growing, rapidly after translocation (Nei *et al.* 1975) and that the genetic

representation of the founders is balanced, from which we can infer that most individuals translocated managed to breed. The risk of loss of further diversity, although always present in populations of finite size (Taylor & Jamieson 2008), is perhaps less concerning than first thought in the Seychelles warbler.

Chapter 3 also added novel data to the growing body of evidence on the relative importance of drift and selection in shaping adaptive variation in natural populations. I showed that microsatellite and MHC differentiation were highly positively correlated. This suggests neutral processes – in this instance founder effects caused by incomplete genetic capture during translocation – are the main forces shaping diversity in small populations, a finding supported by various other studies of neutral and adaptive variation (e.g. Kimura 1983; Seddon & Ellegren 2004; Miller *et al.* 2010; Agudo *et al.* 2011; Sutton *et al.* 2011).

In terms of species management, Chapters 2 and 3 collectively suggest there may be cause for assisted gene flow between the populations established by translocation. This can help improve long-term viability prospects of translocated populations (Lubow 1996; Armstrong & Ewen 2001; Ewen *et al.* 2012) by ensuring genetic diversity is maintained (and well-distributed) across populations that would otherwise remain isolated (Weeks *et al.* 2011). I provided estimates of effective population sizes for each population (ranging from 16 – 82, depending on island and method used; Chapters 2 & 3). These estimates mostly fall well below the recommended minimum viable effective population sizes (50-500, up to 5000; Lande 1995; Franklin & Frankham 1998; Willi *et al.* 2006). When effective population sizes are small, even alleles with strong favourable effects behave effectively neutrally ($s \leq 1/2N_e$; Whitlock 2000; Ferrière *et al.* 2004). In the Seychelles warbler, this means that even strong phenotypic effects, such as the survival advantage conferred by the *Ase-ua4* MHC allele investigated in Chapter 6, may not be propagated in the population. Importantly, this also means that deleterious mutations, equally influenced by stochastic processes, may not be selected out and thus can increase the mutational load a population suffers, leading to extinction vortices (Allendorf & Luikart 2007; Frankham *et al.* 2010). This further adds to concerns about the long-term evolutionary potential, or 'evolvability', of the species and strengthens the argument for assisted gene flow between populations in future, to maximise the overall effective population size of the species.

From a practical perspective, Chapters 3 and 4 provide conservation managers with novel and, I hope, useful data on the experiences and conservation outcomes of 23-years of translocations and post-translocation monitoring. The Seychelles warbler system presents an

unusual case study where there has been no mortality during or immediately following translocation, which is markedly different from other programmes (e.g. Slough 1989; Musil *et al.* 1993; Taylor 2006; Emslie *et al.* 2009). A study by Taylor (2006) identified time in captivity as a major predictor of mortality in translocations of New Zealand saddlebacks. For all Seychelles warbler translocations, a rapid capture and hard-release method was used (detailed in Chapter 4), with most individuals in captivity for less than eight hours and many for considerably less (Komdeur 1994; Richardson *et al.* 2006; Chapter 4). Additionally, considerable effort was spent surveying and selecting potential release islands and sites to minimise transition stress. All releases were immediate, without the use of aviaries. Although the Seychelles warbler is clearly a robust species, the methodology we applied (e.g. practical logistics, catching regimes and surveying) will be relevant to translocations of other species, not just in the Seychelles but globally. Hopefully others will consider using such a 'light touch' approach in their translocation programmes.

7.1.2 Is MHC diversity important to Seychelles warblers?

One interesting consideration is the importance that MHC genes play in the long-term viability of the Seychelles warbler. Throughout diverse areas of evolutionary, ecological and medical research, MHC genes have long served as paradigm of adaptive variation (Beck *et al.* 1999; Penn & Potts 1999; Bernatchez & Landry 2003; Parham 2005; Sommer 2005; Piertney & Oliver 2006; Spurgin & Richardson 2010). They are unquestionably important from an evolutionary conservation perspective, as pathogens are a significant evolutionary force and can pose a substantial extinction risk in isolated or small populations (Hamilton & Zuk 1982; Sheldon & Verhulst 1996; Jones *et al.* 2008; Smith *et al.* 2009; Ohlberger *et al.* 2011). Indeed, MHC class I diversity has been maintained in the Seychelles warbler (Hansson & Richardson 2005) despite the severe bottleneck revealed in Chapter 2, suggesting it has some importance. We have evidence that MHC is important in improving offspring survival as a result of biased extra-pair fertilisations (Richardson *et al.* 2005) and that survival is partially dependent on MHC diversity in young warblers (Brouwer *et al.* 2010). Furthermore, we now have evidence that the single-allele *Ase-ua4* survival effect documented by Brouwer *et al.* (2010) is widespread throughout the population over time, at least on Cousin Island (Chapter 6). However, when investigating social mate choice in two translocated populations of the Seychelles warbler (Chapter 5), I found that male age and neutral heterozygosity predicted male pair status, but the MHC did not. This suggests that social mate choice is not an evolutionary mechanism maintaining MHC diversity in the species, and that we may need to look to other mechanisms (such as male-male competition, forced copulations or post-copulatory mate choice) to explain the patterns of MHC-based extra-pair fertilisations observed (Richardson *et al.* 2005). Collectively, this

suggests that MHC genes do play an important, if complex, role in the evolutionary ecology of the Seychelles warbler.

Whether the MHC diversity remaining in the Seychelles warbler will provide adequate resilience against any novel pathogens that may enter the populations in the future is unknown. Evidence that low MHC diversity reduces population viability in natural populations is equivocal (Radwan *et al.* 2010) and, as discussed in Chapter 3, many species show apparent viability despite low MHC diversity (Slade 1992; Miller & Lambert 2004; Castro-Prieto *et al.* 2011; Gangoso *et al.* 2012). This may be due to a publication bias towards successful conservation outcomes (Radwan *et al.* 2010). Obviously conservation biologists would never characterise the MHC of a species and willingly allow its extinction to test whether this would be the case. Indeed, many of these populations are subject to active conservation effort which may artificially mitigate some risks. However, the risk posed by novel diseases to isolated island populations should not be underestimated (Smith *et al.* 2006; Smith *et al.* 2009). It might also be the case that many of these apparently viable populations are simply yet to encounter novel pathogens that they are unable to combat. The main point is that our assessment of 'survival' is often very short term in scope, which makes gauging 'evolvability' a difficult task.

In Chapter 3, I provide evidence that we successfully translocated all known MHC class I variation to all four new populations. In terms of long-term viability, this is the best-case scenario where we have maximised both neutral and MHC diversity across populations. However, in Chapter 1 I outlined the limitations inherent in using a single gene complex to characterise what is essentially a complex interplay of many adaptive genes across the genome. Although we have come a long way from basing entire genetic management programmes around this single gene complex (Hughes 1991; Miller & Hedrick 1991), we still do not yet understand the interactions between various component genes and molecules of the immune system, and must acknowledge that we may have lost variation in other important adaptive genes in the bottleneck and perhaps failed to distribute the remaining variation as evenly as possible in the translocations. Continuous monitoring of the populations as part of long-term conservation will help identify any problems in the continued recovery of this species (Richardson 2001; BirdLife International 2014). Of particular relevance is the continued surveillance for novel pathogens entering the populations (Hutchings 2009). Such surveillance is routinely carried out as part of the monitoring of this species by the Seychelles Warbler Research Group (consisting of researchers from University of East Anglia, University of Sheffield and Rijksuniversiteit Groningen, in collaboration with Nature Seychelles).

7.1.3 General conclusions & further work

An obvious avenue for future research is to extend the candidate gene approach to other component genes of the immune system. For example, the killer-cell immunoglobulin-like receptors (KIRs) and toll-like receptors (TLRs) are equally significant components of immunocompetence (Belvin & Anderson 1996; Downing *et al.* 2010; Ekblom *et al.* 2010). Studies on population genetics of TLRs in natural bird populations are now underway (Grueber *et al.* 2013), and in the Seychelles warbler work has begun characterising these and other immunity genes (D. Gilroy, unpublished data). Investigating interactions between these genes, the MHC and fitness traits (e.g. Parham 2005) will enable us to further elucidate the role adaptive genetic variation plays in species such as the Seychelles warbler that exist in bottlenecked, isolated and small populations.

Genomic technologies offer unprecedented opportunities for exploring, with increasing speed and economy, the structure and function of MHC and indeed whole genomes, and are rapidly overtaking the use of conformational techniques such as RSCA (e.g. Babik 2010; Metzker 2010; Haase *et al.* 2013; Dudley *et al.* 2014). One promising method for investigating the MHC is ultra-deep sequencing using the Illumina platform. Lighten *et al.* (2014) applied this method to MHC class II genes in a population of guppies (*Poecilia reticulata*) and were able to detect allele-sharing between loci and show evidence of individual copy number variation in this species. This method also boasts a high sequencing repeatability, which is a problem with many next-generation technologies (Amos *et al.* 2010). Application of such techniques to the Seychelles warbler would enable us to further tease apart the structure of the MHC, improve our understanding of the inheritance of alleles and maintenance of MHC diversity, and further clarify its role in the Seychelles warblers' evolutionary ecology. For example, the approach would be helpful in investigating the mechanistic basis of the survival advantage investigated in Chapter 6.

The wealth of samples, life history data and environmental monitoring that the Seychelles Warbler Research Group has accumulated over more than 25 years of research make it a rare, invaluable and exciting model for asking questions in evolutionary conservation (Komdeur 1991; Komdeur & Pels 2005; Clutton-Brock & Sheldon 2010; Hammers *et al.* 2012; Barrett *et al.* 2013). By using this system, I have been able to provide novel data to improve our understanding of how evolutionary history and conservation action interact to shape neutral and functional diversity in natural populations. In doing so, I have addressed some of the challenges identified in evolutionary conservation research by, for example: documenting the effects of bottleneck and translocation events on neutral and functional genetic variation;

integrating genetic, behavioural, ecological and historical data in understanding evolutionary history and contemporary population characteristics; and disseminating the conservation outcomes of valuable long-term monitoring and study of natural populations (Pertoldi *et al.* 2007; Clutton-Brock & Sheldon 2010; Frankham 2010). Although the Seychelles warbler is in some ways unusual, for example in lacking adult predation and dispersal (Komdeur 1991; Komdeur *et al.* 2004), it shares a history typical of endangered species across the globe, with small and isolated populations characterised by significant population decreases in the last hundred years or so (Collar & Stuart 1985; IUCN 2013). With this in mind, it is my sincere hope that the research presented in this thesis may be useful in understanding and conserving biodiversity on a wider scale and serve as an example of the relevance of evolutionary conservation in pursuit of that goal.

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