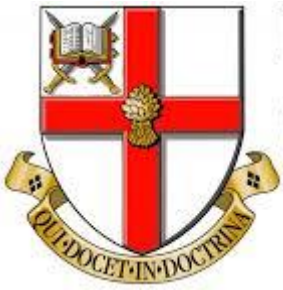

University of Chester

Application of genetic techniques to conservation of the Critically Endangered Grenada Dove *Leptotila wellsi*

Thesis submitted in accordance with the requirements of the University of Chester for the degree of Doctor of Philosophy

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in collaboration with the Forestry and National Parks Department of the Government of Grenada and the Grenada Dove Conservation Programme

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Declaration

"The material being presented for examination is my own work and has not been submitted for an award of this or another HEI except in minor particulars which are explicitly noted in the body of this thesis. Where research pertaining to the thesis was undertaken collaboratively, the nature and extent of my individual contribution has been made explicit."

Signed: 

Date: 30th September 2022

Abstract

The Grenada Dove *Leptotila wellsi* is a Critically Endangered species endemic to the island of Grenada. It exists in two geographically isolated populations across the island. As an endemic on a small island with a limited and decreasing population, this species is at risk from genetic deterioration. Particular risks include inbreeding depression and accelerated loss of genetic diversity resulting from ongoing habitat fragmentation and population isolation. The work reported in this thesis aimed to provide the first genetic data for the Grenada Dove, assess its genetic status and determine what conservation management recommendations can be made to help ensure its long-term survival. A systematic literature review revealed geographical bias in how genetic approaches have been applied to the conservation of island bird species, under-representation of threatened species and highlighted a gap between genetic research and active conservation management. A protocol for obtaining usable genetic material from moulted plumulaceous feather samples was developed and showed that samples collected non-invasively in the field, that were kept in non-optimal conditions for deoxyribonucleic acid (DNA) extraction, can be used effectively to sequence informative regions of mitochondrial DNA (mtDNA) for genetic analysis. The evolutionary and life history of the Grenada Dove were investigated by estimating its phylogenetic placement and using phylogenetic comparative methods for estimating unknown life history traits. The Grey-Chested Dove *Leptotila cassinii* was identified as the species with which the Grenada Dove shares the most recent common ancestor, with an estimated divergence of approximately 2.53 million years ago. Life history trait values were predicted for the Grenada Dove and suggest this species will be slow to recover after a major population decline. The mitochondrial genetic diversity of Grenada Dove populations was assessed for the two areas of occupancy. Two haplotypes were identified with one haplotype unique to the population in the West. Selective neutrality tests Fu's *Fs*=0.78 and Tajima's *D*=0.83 were positive and not significant, with an F_{ST} value = 0.71 suggesting a marked genetic divergence between the two populations. This study showed low mitochondrial genetic diversity, a non-expanding population and

clear evidence for genetically isolated populations. Population viability analysis (PVA) was used to evaluate extinction risk and the potential for conservation management for the two populations of the Grenada Dove. This PVA identified that loss of forest habitat to commercial development is likely to have the biggest impact on extant populations and that conservation management to increase productivity is likely be the most effective conservation strategy. This thesis contributes novel information regarding evolutionary history, life history, population genetics and future population trajectories to inform long-term conservation actions for the Critically Endangered Grenada Dove.

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CHAPTER 1 INTRODUCTION

Photo by Alice McCourt

Chapter 1: General Introduction

1.1 Introduction

The Grenada Dove *Leptotila wellsi* is a Critically Endangered island endemic bird species which is at a high risk of extinction (BirdLife International, 2021b, 2022b; Rusk, 2017). The genetic status of this species is unknown, and due to its small and fragmented populations, genetic factors are likely contributing to its probability of extinction. Conservation of this species, guided by novel genetic information, will contribute to securing its long-term survival.

1.1.1 Avian Biodiversity Loss and Extinction

The last 100 years has seen the extinction of around 400 vertebrate species (Ceballos *et al.* 2020) and life has now entered what many consider to be a sixth mass extinction (Ceballos *et al.* 2015, 2020; Spatz *et al.* 2017; Díaz *et al.* 2019). The decline of vertebrate species is particularly high on islands which are widely considered hotspots of threatened species and extinction (Ceballos *et al*. 2017; Frankham *et al*. 2002). Island losses account for 53.8% of mammal, 81% of reptile, 53.8% of amphibian and 95.3% of bird species listed by the International Union for the Conservation of Nature (IUCN) as extinct (Tershy *et al.* 2015). Birds are the best studied class of organisms, particularly when it comes to extinctions and are also the most species-rich clade of terrestrial vertebrates (Butchart *et al*. 2018; Lees *et al*. 2022). While there is a global decline of bird species, threatened birds appear to be largely concentrated in the tropics (Ceballos *et al.* 2017; Lees *et al.* 2022). Around 224 birds are thought to be Critically Endangered and it is presumed that approximately 10% of these species are already extinct (Carmona-Isunza *et al.* 2020; IUCN 2020). The rate of avian extinctions have been have been increasing since the 1500's (Butchart *et al.* 2018; Lees *et al.* 2022) with a number of bird species, including the Aldabra Warbler *Nesillas aldabrana*, Guam Flycatcher *Myiagra freycineti*, and the Atitlán Grebe *Podilymbus gigas* becoming extinct in just the last 40 years, and some, such as the Cryptic Tree Hunter *Cichlocolaptes mazarbarnetti*, Angoas Foliage-gleaner *Philydor novaesi* and the Poo'uli *Melamprosops phaeosoma,* declared extinct as recently as the 2010's (Hume and Walters 2012; Szabo

et al. 2012; Butchart *et al.* 2018). Recent extinctions are difficult to detect as often locations with high extinction rates are isolated and understudied, consequently, increased effort to reduce significant threats to avian biodiversity and greater conservation interventions are required to prevent further bird extinctions, particularly for birds in the tropics which are at the highest risk (Lees *et al*. 2022).

1.1.2 Causes of Avian Extinction

The main reasons for loss of biodiversity are known as the "evil quartet" and are: habitat fragmentation, over exploitation, alien invasive species, and co-extinction (Diamond, 1984; Godet & Devictor, 2018). The primary avian extinction risk, particularly for insular bird species, is the introduction of invasive species (Butchart *et al.* 2018; Lees *et al.* 2022). Introduced mammals were the foremost historical cause of avian extinction and are still a significant contemporary threat to extant bird populations (Lees *et al*. 2022). The consequences of invasive species to affected bird populations include increased predators and competition for resources (Clavero & García-Berthou, 2005; Jamieson, 2007; Parlato *et al.* 2015) and in some cases, can cause modifications to habitat and even ecosystem collapse (Lees *et al.* 2022; Morris-Pocock *et al.* 2012; Showler *et al.* 2002). The introduction and enhanced transmission of disease is an additional threat caused by invasive species (particularly introduced domesticated birds) with outbreaks driving declines in a large number of avian species (Wikelski *et al.* 2004; Thomas *et al.*2007; Lees *et al.* 2022). Hunting and trapping of birds, while a direct threat, also has indirect effects on ecosystems as the removal of birds impacts upon seed dispersal, predation and ultimately causes habitat degradation (Dimitriou *et al.* 2017; Butchart *et al.* 2018; Lees *et al.* 2022).

Habitat loss and degradation via climate change, residential and commercial development, and land-use change are major drivers of biodiversity loss, restricting bird species to small habitat patches, affecting dispersal and increasing the risk of local extinction (Frankham *et al.* 2017; Butchart *et al.* 2018; Lees *et al.* 2022). For many species extinction risk is multi-faceted and birds often face a combination of the threats discussed above. The Critically Endangered Madagascan Pochard *Aythya innotata,* for example, is threatened by alien invasive species, hunting and trapping, loss of habitat

and, due to its extremely small population size, faces significant risk from genetic factors such as inbreeding depression; without conservation intervention, extinction is a distinct probability for this species (Donald *et al.* 2010; BirdLife International 2022a). However, having robust knowledge of species specific extinction risks and expanding on conservation efforts which take into consideration the long-term viability of populations (i.e. effective population size, habitat availability, genetic diversity), such island species which are facing multiple threats may still be saved (Donald *et al.* 2010; Jackson *et al.* 2022; Lees *et al.* 2022).

1.1.3 Genetics Factors involved in Extinction

The role of genetics in extinction has been explored and documented over the last three decades (Lande 1988; Frankham 2005; Allendorf *et al.* 2007; Jamieson 2007). Genetic factors such as inbreeding and loss of genetic diversity increase the probability of extinction particularly in small populations (Frankham, 2005; Taylor & Jamieson, 2008). Evidence shows that threatened species and island endemics have lower genetic diversity when compared to taxonomically related non-threatened taxa (Frankham, 1998; Jamieson, 2007); they are also more prone to inbreeding depression, bottlenecks and genetic drift (Frankham 1997; Grant *et al.* 2001; Dudaniec *et al.* 2011; Gonzalez-Quevedo *et al.* 2015). Inbreeding depression – the reduction in biological fitness of a population - occurs in small, isolated mating populations, and is often greater in stressful environments (Armbruster & Reed, 2005; Frankham *et al.* 2019; Hedrick & Kalinowski, 2000). It has been known to reduce reproductive fitness, increase homozygosity and the proportion of harmful recessive alleles, and reduce the heterozygote advantage - where heterozygotes have a higher relative fitness than homozygotes (Frankham *et al.* 2010, 2019).

Inbreeding and loss of genetic diversity can reduce the adaptability of a species to evolve in response to environmental change and can decrease resistance to diseases and parasites (Spielman *et al.* 2004; Frankham *et al.* 2010, 2019). The evolutionary adaptive potential of a species is reliant upon genetic diversity, selection and effective population size, all of which are often reduced in small isolated populations (Allendorf *et al.* 2007; Frankham *et al.* 2010, 2019). Given the accelerated rate of

extinction, reducing inbreeding and negating the loss of genetic diversity are imperative conservation measures for safeguarding a species' evolutionary potential and adaptive fitness (Ficetola and Bonin 2011; Frankham *et al.* 2017; Mable 2019).

1.1.4 What is Conservation Genetics?

Conservation genetics is a discipline which applies genetic theory and practical techniques to conservation biology problems (Frankham et al., 2010; Hedrick & Miller, 1992; Hedrick, 2001). This discipline is predominately focused on increasing knowledge of genetic consequences arising from low genetic diversity and inbreeding, and using this information to reduce the risk of extinction in threatened species (Frankham *et al.* 2010). Genetic research can be used to aid species conservation in a variety of ways, such as: hybridization detection; identification of illegal hunting and collection; and defining evolutionarily significant management units (ESUs) (Frankham *et al.* 2010). With new and advancing technologies, genetic studies have become widely used in conservation biology research, providing a better understanding of genetic processes along with increasing recommendation of appropriate management of threatened species (Frankham et al., 2019; Wallis, 2019; Willi et al., 2022).

1.1.5 Genetic Management of Threatened Species

Genetic management aims to minimise the negative consequences associated with low genetic diversity. Techniques include increasing the rate of gene flow e.g. translocation, creation of habitat corridors and population augmentation by re-establishment of populations in suitable habitat (Lawrence *et al.* 2014; Khimoun *et al.* 2017; Talbot *et al.* 2017), genetic purging to remove deleterious alleles from a population (Laws and Jamieson 2011; Robinson *et al.* 2018) and conservation breeding programmes to manage populations which may not survive in the wild (Williams and Hoffman 2009; Frankham *et al.* 2010, 2017, 2019). Captive breeding (also referred to as conservation breeding) is perhaps the most well-known conservation management strategy and frequently uses pedigrees and genetic testing to promote outbreeding, aiming to increase both population numbers and genetic

diversity (Williams and Hoffman 2009; Dolman *et al.* 2015; Collar 2020). This approach has had success, particularly in small populations where bottlenecks have caused loss of genetic variation (Bowker-Wright *et al.* 2012; Major *et al.* 2021). In the case of the Critically Endangered California Condor *Gymnogyps californianus,* for example*,* populations were managed to prevent decline of genetic diversity using the concept of mean kinship to identify genetically important individuals, and resulted in a more favourable genetic status with a lower number of related individuals when compared to other captive-bred species (Ralls & Ballou, 2004)**.**

Before making genetic management decisions, there are a number of factors which need to considered, such as: whether the taxonomy is reliable enough to define management units; whether populations are genetically isolated; and how best manage gene flow (Jones *et al.* 2006; Frankham *et al.* 2017). Both demographic (increasing population size) and genetic (facilitating adaptation and reducing inbreeding) management actions need to be carefully selected and monitored in order to avoid inadvertent genetic consequences; for instance, homogenising existing diversity and over simplifying evolutionary lineages (gene pools) can lead to introgression (often following hybridisation) which threatens the genetic integrity of a species (Allendorf *et al.* 2007; Frankham *et al.* 2017; Gippoliti *et al.* 2018; Hufbauer *et al.* 2015). The Vulnerable Echo Parakeet *Psittacula eques* was managed using translocations, however, an increase in productivity of wild breeding pairs was the priority rather than genetic representation when selecting individuals for translocation (Raisin *et al.* 2012). Raisin *et al.* (2012) assessed the genetic structure of the Echo Parakeet and revealed that before intensive management populations showed a clear signal of structure, however, this spatial structure disappeared after intensive conservation management. This highlights how spatial patterns of genetic diversity can be influenced by intensive conservation management and how translocations, when managed effectively to included unrelated individuals, can reduce the of loss of genetic diversity.

Genetic rescue – outcrossing of a population showing signs of inbreeding and low genetic diversity to improve reproductive fitness and increase genetic diversity – is a management strategy used in the recovery of small inbred populations (Frankham *et al.* 2010). One species which benefited

from genetic rescue measures was the Pink Pigeon *Nesoenas mayeri;* a species endemic to Mauritius (Swinnerton *et al.* 2004; Jackson *et al.* 2022). Intensive conservation management (including captive-breeding, translocations, supplemental feeding and predator control) instigated a rapid population recovery from c.10 individuals in 1990 to more than 400 in 2013, resulting in a conservation status down-listing on the IUCN Red List of Threatened Species (Jones 2013; Copsey *et al.* 2018). Jackson *et al.* (2022) analysed the impacts of genetic rescue during and after population recovery and conducted population viability analysis (PVA) to predict future genetic impacts; the results showed that of the four scenarios (no intervention, demographic rescue, genetic rescue, demographic + genetic rescue) the scenario which combined both demographic and genetic rescue had the lowest extinction proportion and hence was the best management strategy. This study stressed that further genetic rescue is imperative to recover lost variation and reduce expression of harmful deleterious variation, and that incorporation of genetic management can improve conservation management decisions to ultimately prevent extinction (Jackson *et al.* 2022). As such, incorporating genetic measures into conservation actions can not only contribute to a species recovery but also helps in securing the long-term viability of populations (Raisin *et al.* 2012).

1.1.6 Sampling Techniques for Genetic Analysis

Given advances in molecular methods, a variety of sample types are now being used for genetic research, however, recent genomic approaches require samples that will yield high quality deoxyribonucleic acid (DNA) (Carroll *et al.* 2018). Hence, sampling methods can affect the scale and content of data collected (Segelbacher *et al.* 2010). Invasive sampling is a method which results in the alteration of the condition of the individual being sampled (Taberlet & Luikart, 1999). Examples include blood (van de Crommenacker *et al.* 2015) and tissue samples (Taylor *et al.* 2018), with both typically providing high quality template data (Zemanova, 2021). Minimally/ partially invasive sampling such as buccal swabbing (Phillips *et al.* 2020), feather (Stoeckle *et al.* 2012) and hair plucking (Hu *et al.* 2011), while less intrusive, often still require trapping and handling of individuals (Peters *et al.* 2019). Strictly non-invasive sampling, however, is a method of obtaining samples without capturing

or even observing the individual (Taberlet *et al.* 1999). Examples include egg shells (Urano *et al.* 2011), moulted feathers (Ramón-Laca *et al.* 2018), faecal samples (Ang *et al.* 2019) and environmental DNA (eDNA) (Belle *et al.* 2019). Non-invasive sampling can make species identification difficult and result in poor sample quality, affecting the utility of such samples for genetic analysis; however, non-invasive sampling may be the only opportunity to obtain samples for rare or elusive species (Mills *et al.* 2000; Peters *et al.* 2019). Obtaining approvals for intrusive sampling methods of endangered species, accessing isolated and hard-to-reach habitat, tracking down species with low encounter rates and physically capturing species makes obtaining samples logistically difficult; as such, the use of non-invasive methods makes genetic sampling in these circumstances still possible (Segelbacher 2002; Peters *et al.* 2019; Zemanova 2021). Non-invasive sampling is also considered more ethically acceptable, particularly when working with threatened species, as it reduces negative implications for animal welfare. Given improved methods to extract DNA, the increased possible application of such samples is prompting many wildlife managers to shift to this approach (Segelbacher, 2002; Zemanova, 2021).

Prior to conducting genetic studies a variety of factors should be considered to aid in choosing an appropriate sampling method, for example: the conservation status of the species, the sample source (museum, wild, captive) and the sample type, which can affect the type of investigation that can be carried out and the inferences that can be made (Segelbacher *et al.* 2010; Carroll *et al.* 2018; Zemanova 2021). Samples from museum specimens for instance, can be valuable resources for species identification or historical diversity assessments but cannot give insights into the genetic diversity within contemporary populations (Freeland *et al.* 2007; Carroll *et al.* 2018). Grueber and Jamieson (2011) conducted genetic analysis on the Endangered Takahē *Porphyrio hochstetteri* using toe-pad museum samples alongside blood samples from contemporary populations (one of which had been supplemented with captive bred-birds), enabling it to shed light on historical diversity while also making management recommendations to prevent further loss of genetic diversity.

1.1.7 Applied Conservation Genetics

Genetic knowledge of species and populations can be applied to conservation in an assortment of ways (Frankham *et al.* 2010). Due to the overwhelming number of plants, animals, and fungi he taxonomic status of a considerable number of species remains unknown, therefore, DNA barcoding methods have been developed to classify a broad range of species and can be used in conjunction with phenetic-based classification systems in order to resolve such taxonomic uncertainties (Kress *et al.* 2015; Mable 2019). In the case of Crowned Pigeons (Columbidae*: Goura*), genomic data was used to support proposed taxonomic revisions from three to four species with implications for the conservation status of species within this genus (del Hoyo and Collar 2015; Bruxaux *et al.* 2018). Additionally, genetic methods for species identification can be used to provide information in wildlife forensic cases, particularly in cases of illegal trade when a species cannot be identified from seized evidence (Alacs *et al.* 2009). The illegal trapping, trading and consumption of small songbirds in Cyrpus is widespread, to combat this a DNA sequence database (comprising of the cytochrome oxidase subunit 1 (*COI*) barcoding region for birds) of all bird species resident in Cyprus was generated to assist wildlife officials with identification of species from incomplete, deformed or cooked samples, thus aiding prosecutions (Dimitriou *et al.* 2017). Molecular methods can also be used to identify the sex of an individual (Çakmak *et al.* 2017). This is particularly useful for monomorphic bird species which are often difficult to sex, and is especially valuable in captive breeding programmes when choosing which birds to house together (Frankham *et al.* 2010). One monomorphic species is the Japanese Crested Ibis *Nipponia nippon* and is the subject of a captive-breeding programme (Kasuga *et al.* 2012). Kasuga *et al.* (2012) validated a Polymerase Chain Reaction (PCR) based sexing method for accurate sexing of captive-bred individuals to aid research following the release of birds into the wild.

Phylogenetic methods can be used to reconstruct informative relationships within genus and family groups (Johnson *et al.* 2001), reveal evidence for speciation and evolutionary history (Johnson & Clayton, 2000b), and identify isolated lineages (Robin *et al.* 2010). A study using mitochondrial and

microsatellite DNA markers along with morphological data was able to provide the first insight into the evolutionary, genetic and morphological differentiation of the Seychelles Black Parrot *Coracopsis nigra barklyi,* and recommended that management as an ESU will help to conserve its unique evolutionary pathway (Jackson *et al.* 2016). Phylogenetic comparative methods, is another use of genetic data which combine phylogenetic data with demographic and ecological data to fill knowledge gaps, for example, James *et al.* (2020) used phylogenetic imputation to calculate missing demographic metrics such as generation time, age at maturity and lifespan in birds highlighting the use of these methods as a conservation tool for imputing vital rates for species of conservation concern when field data are not available.

Analysing genetics at a population level can be used to reduce extinction risk by assessing inbreeding levels and genetic variation, and recommending management actions to minimise loss of genetic diversity (Frankham *et al.* 2010). Population genetics can also be useful in evaluating population connectivity and identifying isolated population which are at risk of reduced genetic diversity (Prugh *et al.* 2008; Alcaide *et al.* 2009; Frankham *et al.* 2010). Examining gene flow can be used to assess connectivity, identify fragmentation and reveal genetic differentiation among populations (Lindsay *et al.* 2008; Frankham *et al.* 2010; Nogueira *et al.* 2014). The Plumbeous Warbler *Setophaga plumbea* was the focus of a landscape genetic study which revealed a reduction in gene flow between populations as a result of forest fragmentation (Khimoun *et al.* 2017). Rather than investigating gene flow between populations of a single species, Booth Jones *et al.* (2017) explored the possibility of between-species hybridization, and demonstrated widespread gene flow between a group of *Pterodroma* petrels, highlighting the importance of awareness of migration and hybridization in the conservation of threatened seabirds. Ultimately, all conservation genetic studies contribute to a greater understanding of a species' biology and ecology which is lacking for many poorly understood species. This increased knowledge can be used to guide conservation actions which may prove crucial for many threatened species, particularly island endemic species whose often small populations are

at a higher risk of isolation, inbreeding depression and ultimately extinction (Frankham, 2005; Frankham *et al.* 2010; Taylor & Jamieson, 2008).

1.2 Grenada Dove *Leptotila wellsi*

1.2.1 Systematics and Identification

Pigeons and doves (Columbiformes; Columbidae) are one of the most widespread lineages of birds inhabiting six of the seven continents (Pereira *et al.* 2007; Soares *et al.* 2016). Adaption to a wide range of ecological environments has led to Columbiformes being one of the most diverse of all the avian orders, an order which consists of approximately 348 species (Soares *et al.* 2016; Winkler *et al.* 2020). Despite this the evolutionary history of this group is still poorly resolved (Pereira *et al.* 2007; Soares *et al.* 2016).

The Grenada Dove *Leptotila wellsi* is a Neotropical Columbid*,* of the subfamily *Leptotilinae*, (Whitlock 1981; Rusk 2017; Baptista *et al.* 2020). While considered a monotypic species, it belongs to the genus *Leptotila* which is comprised of 11 morphologically similar species (Gibbs *et al.* 2010; Winkler *et al.* 2020), and in the past it has been considered conspecific with the Grey-fronted Dove *L. rufaxilla ,* Grey-headed Dove *L.plumbeiceps,* Azuero Dove *L. battyi* and Pallid Dove *L. pallida* (Baptista *et al.* 2020).

The species name *wellsi* originates from the first documented specimen of this species collected by J. G. Wells in 1884. George Newbold Lawrence writes of correspondence between himself and J. G. Wells in an article entitled '*Characters of a new species of pigeon of the genus Engyptila, from the island of Grenada, West Indies'* regarding a list of birds of Grenada which Wells intended to publish (Lawrence, 1884). Whilst compiling the list Wells would send bird skins through the Smithsonian Institution to Lawrence for identification (Wells, 1886). This included a skin from a species that Wells termed the "Pea dove" and led to the description of a new species, the Grenada Dove, being documented (Lawrence, 1884). Wells went on to publish the list entitled "*A catalogue of the birds of Grenada, West Indies, with observations thereon*" in the Proceedings of United States National Museum in 1886 in which the Grenada Dove features with the description "*A beautiful dove, new to*

me, about the size of Z. martinicana; frequents a place called Fontenoy, on the wester side of the island" (Wells, 1886). The original placement of this species, by Lawrence, was in the genus *Engyptila*, hence the first documented name for the Grenada Dove was *Engyptila wellsi* (Lawrence, 1884). The genus *Engyptila* was renamed as *Leptotila* in 1895 (Coues *et al.* 1895).

The Grenada Dove is morphologically similar to other members of the *Leptotila* genus which lead to deliberations amongst scientists as to whether the Grenada Dove was a distinct species as originally documented (Blockstein & Hardy, 1989; Lawrence, 1884). In 1983 the Grenada Dove was reclassified as a sub species of Grey-fronted Dove *L. rufaxilla* by the American Ornithologists' Union (American Ornithologists Union, 1983). However, debates amongst taxonomists continued and in 1989, Blockstein and Hardy presented data based on morphological characteristics and sonography defining the Grenada Dove as a distinct endemic species. This study reported three distinguishing plumage characteristics: colour of the tail tips; abdomen and underwing, between *L. wellsi* and *L. rufaxilla*. It also confirmed the colour of the orbital skin of *L. wellsi* as red and not blue as had been previously reported in error by Schwartz and Klinikowski (1963) and then subsequently by Lack and Lack (1973) and Bond (1973). In addition to the morphological analysis, sonograms were made of the vocalisations of the three species (*L. wellsi*, *L. rufaxilla,* White-tipped Dove *L. verreauxi*) under investigation (Blockstein & Hardy, 1989). All three bird songs showed differences, with the Grenada Dove call being the most distinct of the three. Recordings of the vocalisations of all three species were played in known habitats of the Grenada Dove. Zero responses were observed during 35 playback trials of *L. rufaxilla* and just one response during 35 playback trails of *L. verreauxi;* in contrast a 67% response to playback of *L. wellsi* call was observed. Blockstein and Hardy concluded that the Grenada Dove is most closely related to the allopatric *L. rufaxilla* and is likely to have colonised Grenada from Trinidad, with this reproductive isolation accounting for the differentiation in song and plumage to a level sufficient to conclude that the Grenada Dove be reclassified as a distinct species. The classification of the Grenada Dove as a distinct species was approved in 1995 (Monroe *et al.* 1995). Prior to this study no genetic investigation had been conducted to explore this.

The Grenada Dove (Figure 1.1) varies in size between 28-31cm in length (Raffaele *et al.* 2003; Winkler *et al.* 2020). It is a bicoloured dove with a brown upper body and pale underbody and both its primary and secondary feathers are a plain brown with a reddish-brown underwing. The tail is brown with the rectrices tipped with white, and the breast is a cinnamon colour which grades to a white belly and vent (Raffaele *et al.* 2003; Gibbs *et al.* 2010; Baptista *et al.* 2020). The forehead to crown is a pinkish-white, grading to greyish brown as it reaches the nape. It has a black bill and coral-red tarsi, feet and orbital skin (Gibbs *et al.* 2010). There is no obvious difference between males and females, however the females are reported to be duller with reduced plumage bloom in breeding season (Gibbs *et al.* 2010; Baptista *et al.* 2020). Juveniles are also duller, with dull reddish-purple orbital skin and dull red feet and legs, as well as buff-fringed scapulars and wing-coverts with cinnamon tips to the breast (Baptista *et al.* 2020). The voice of the Grenada Dove is a distinctive, descending "*hoooo*" repeated every 5-8 seconds (Raffaele *et al.* 2003; Baptista *et al.* 2020) see section **1.2.6 Behaviour and Ecology** for more information on vocalisation.

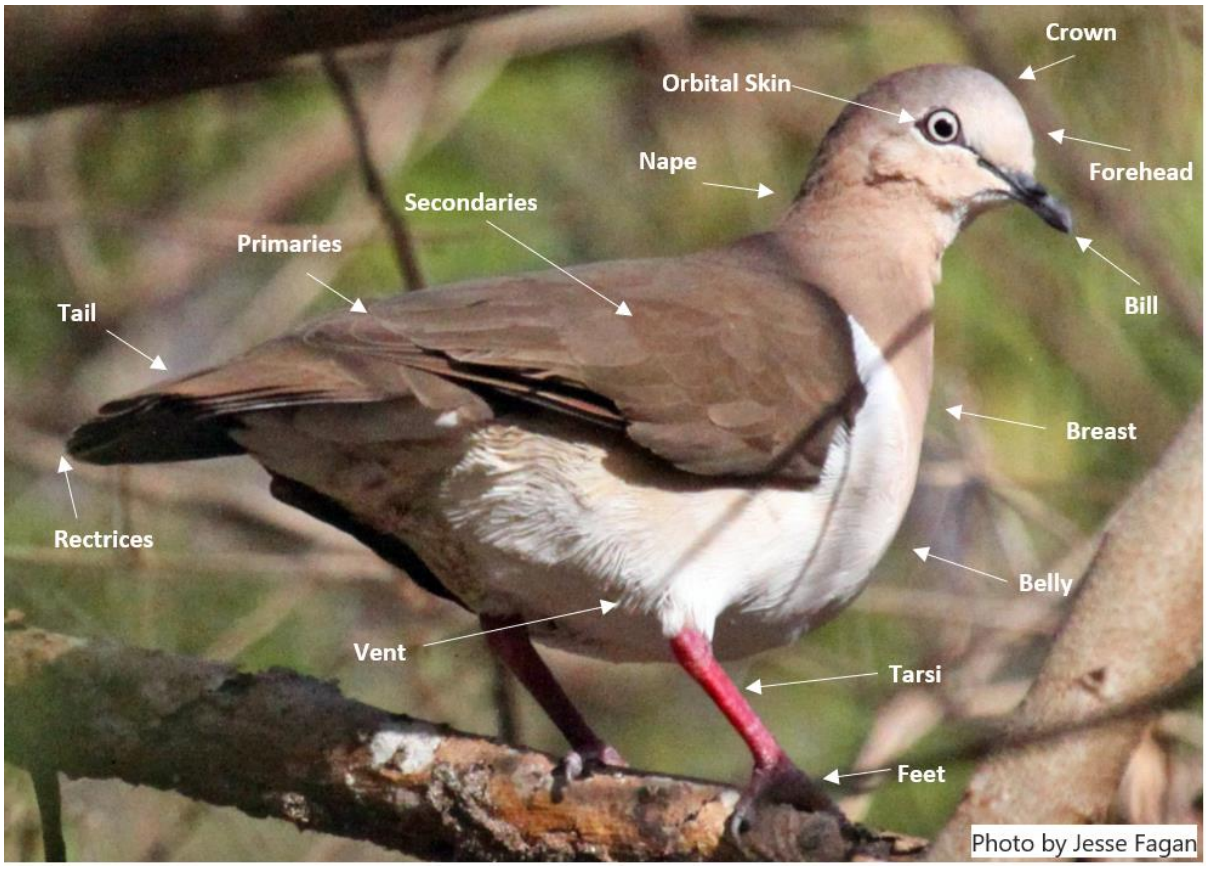

Figure 1.1: Image of the Grenada Dove (Fagan, 2016) adapted with labelled bird topography.

1.2.2 Conservation Status

The Grenada Dove is classified as Critically Endangered by the IUCN Red List of Threatened Species under criterion C2a due to its small population size and continuing decline and was last assessed in March 2021 (BirdLife International, 2021b; IUCN, 2012). The Grenada Dove is legally protected from hunting and egg collection (BirdLife International, 2021b). In addition to legal protections, this species also has cultural significance, with Prime Minster Sir Nicholas Alexander Brathwaite declaring the Grenada Dove the country's national bird on the 15th December 1991 (Butler *et al.* 1992) as discussed in section **1.2.8 Grenada Dove Conservation**.

1.2.3 Geographic Occurrence and Population trends

The Grenada Dove is endemic to the island of Grenada (311km^2) which is situated in the Lesser Antilles of the Caribbean (Figure 1.2) where it is a permanent year-round resident (Rusk, 2009). Extant populations of the Grenada Dove occur in the Southwest and West of the island and are both small and thought to be isolated (Rusk 2009, 2017; Bolton *et al.* 2015; Rivera-Milán *et al.* 2015). The most recent population survey estimated 160±30 individuals (Rivera-Milán *et al.* 2015). The population is thought to be decreasing and it is estimated that it will see a 1-19% reduction over the next 3 generations (BirdLife International, 2021b). A number of Grenada Dove population surveys have been carried out since the 1980s using territory mapping (Blockstein 1988, 1991; Roberts and Taylor, 1988; Rusk 1992, 1998a, 2005, 2008; Rusk and Temple 1995; Rusk and Clouse 2004), point counts and multi-covariate distance sampling (Rivera-Milán *et al.* 2015), along with incidental observations and casual searches (Rusk, 2017).

Across a twenty-year survey period the population trend varies, starting with an initial decrease in the 1980's (Blockstein, 1988; Roberts & Taylor, 1988; Sievert, 1988), then an increase during the 1990's and early 2000's (Rusk, 1998; Rusk & Clouse, 2004). This was then followed by a population decline after a category five hurricane - hurricane Ivan - in 2004 (Rusk, 2005, 2008). The most recent surveys suggest the population has remained relatively stable (Rusk 2008, 2017; Bolton *et al.* 2015; Rivera-Milán *et al.* 2015). This fluctuating population trend may be attributed to a variety

of factors. Initial increases observed may be a reflection of an improvement in survey method techniques (BirdLife International, 2021b) whereas, the decrease following hurricane Ivan is a possible response to increased stress due to habitat and resource loss resulting in a lower number of calling males (Rusk, 2017). Variations of inter-observer reliability could contribute to an increase or decrease in presumed population sizes (Harris & Haskell, 2007). The population trend is listed as decreasing by the IUCN and BirdLife International (BirdLife International, 2021b, 2022b) but while the population may not necessarily be in a clear decline, the increased frequency of hurricanes due to climate change (Walsh *et al.* 2016; Nelson *et al.* 2018), means it is also not likely to recover from major disasters especially when coupled with other threats (BirdLife International, 2022b). In spite of the number of surveys that have been carried out, the population size of the Grenada Dove may in fact be lower than currently reported as the majority of estimates have been calculated based on the number of calling males assuming a 1:1 sex ratio, however, the sex ratio for this species is unknown (Bolton *et al.* 2015). Grenada Dove populations have been small for an extended period and are vulnerable to stochastic events, such as hurricanes. Further information on their genetic diversity, along with empirical evidence for the sex ratio of the population are urgently needed to provide context for these population estimates.

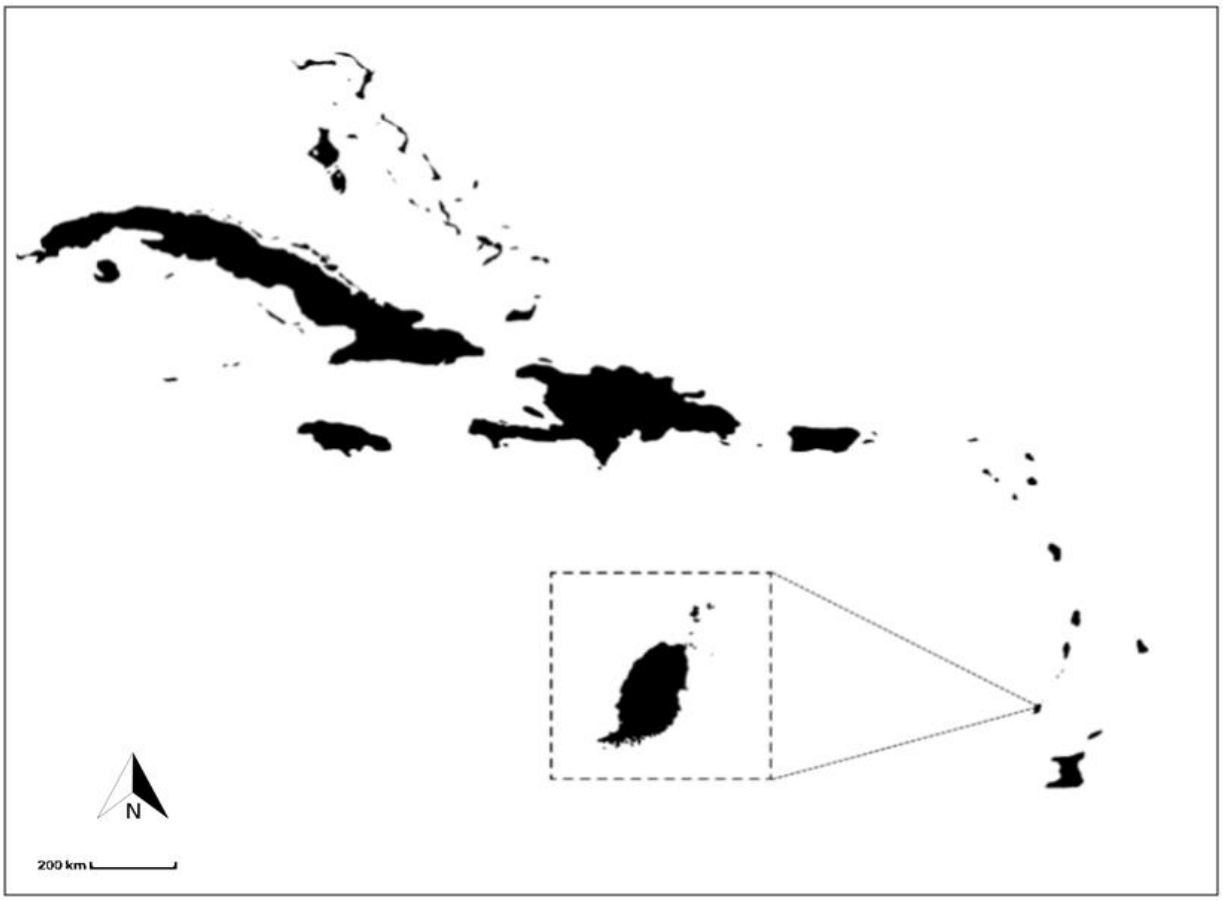

Figure 1.2: Map of the Caribbean drawn for this study using Canva®2022 with map insert highlighting the island of Grenada (Canva®, 2022).

1.2.4 Current Range and Historical distribution

The Grenada Dove exists largely in locations of coastal dry forest in the Southwest and West of the island (Figure 1.3) with the extent of occurrence of approximately 31km^2 (BirdLife International, 2021b, 2022b; Rusk, 2017). In the Southwest this area includes Mount (Mt) Hartman estate, Petit Bouc and Woodlands, Lower Woburn and surrounding forests. In the West this area includes Perseverance, Woodford, Beausejour and Grenville Vale estates and Black Bay (Rusk, 2017). The Southwest and West populations are believed to be isolated due to the 9km^2 of extensive development - including capital city St Georges - which separates the two (BirdLife International, 2021b; Blockstein, 1991; Rusk, 2017). Mt Hartman is perhaps the most important site in the Grenada Dove range and is home to around 37% of the species population (Rusk, 2009). Legal protection was assigned in 1996 at both locations in the form of Mt Hartman National Park (62 ha) and Perseverance Dove Sanctuary and Protected Area

(32 ha) which in total makes up less than 10% of the species range (Bolton *et al.* 2015; Rusk 2017). Although the Grenada Dove inhabits five out of six of Grenada's internationally recognised Important Bird Areas (IBAs), only two of these areas (mentioned above) are legally protected (Rusk 2009; Bolton *et al.* 2015). While there is no evidence of established populations elsewhere on the island, single occurrences have been recorded inland within the parish of St David in the Southeast (Rivera-Milán *et al.* 2015). These additional observations outside of the primary locations in the Southwest and West could indicate recent dispersal of the population to other suitable habitat across the island (Rivera-Milán *et al.* 2015; Rusk 2017).

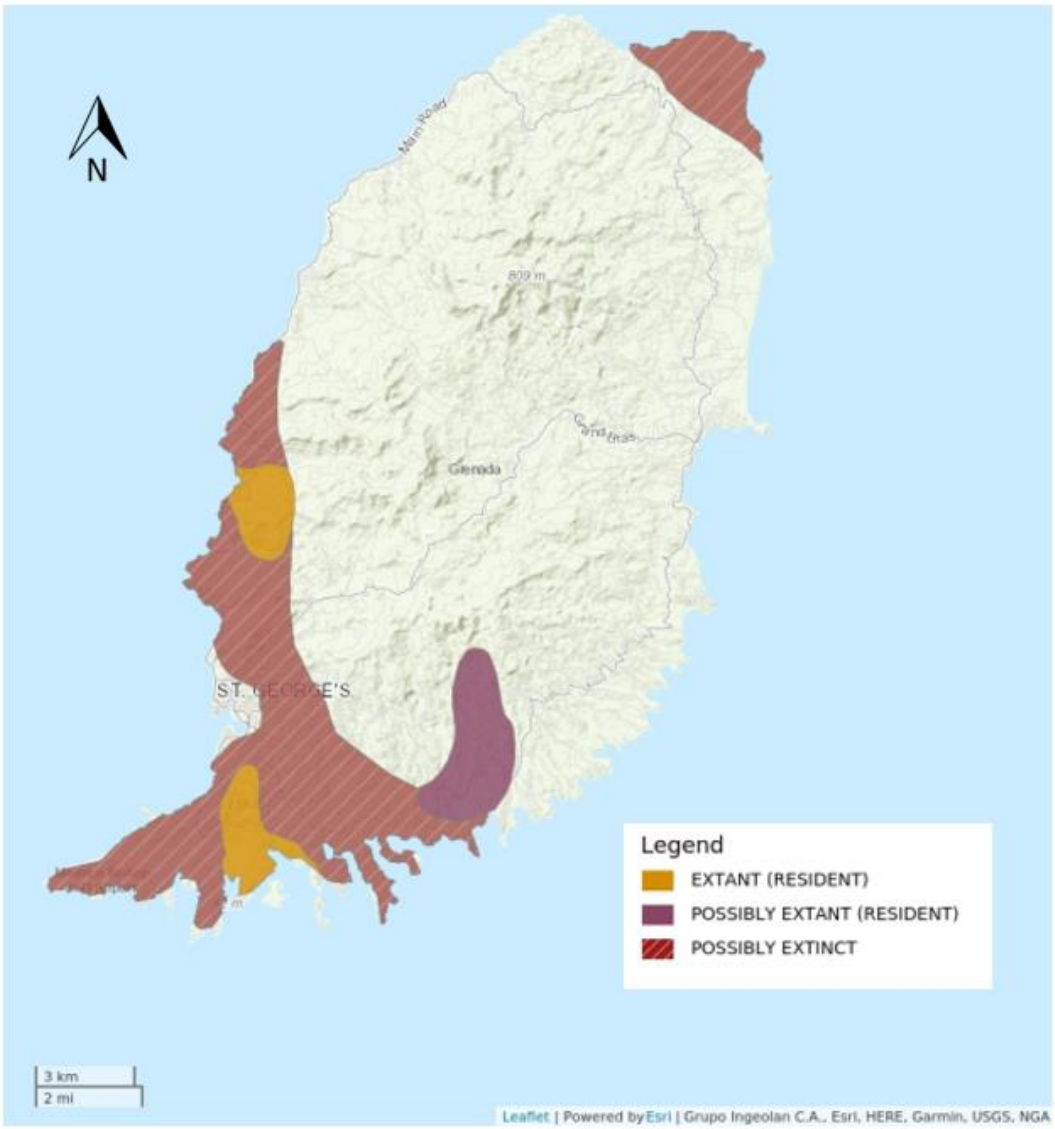

Figure 1.3: Map of Grenada showing the extant and historical range of the Grenada Dove, obtained from The IUCN Red List of Threatened Species (BirdLife International, 2021b) and adapted for this study.

The historical distribution of the Grenada Dove is thought to have once been widespread across dry coastal zones in the Northeast, West and Southwest of Grenada, including off-shore islets (Baptista et al., 2020; Bond, 1973; Clarke, 1905; Collar *et al.*, 1992; Lack & Lack, 1973). In the Northeast this included the coastal dry forest of Levera and the off-shore Green Island (Blockstein, 1988, 1991). Possible historical connectivity between the two extant locations is supported by evidence of Grenada Dove recorded at Fontenoy which lies centrally between the two (Lawrence, 1884). Additional records range from as far South as Point Salines (Blockstein, 1988) and Glover's Island (Schwartz & Klinikowski, 1963; Wells, 1886), to higher locations at Halifax Harbor on the West coast (Lack & Lack, 1973; Rusk, 2017; Wunderle, 1985). The more widespread distribution may have been due to the availability of habitat prior to anthropogenic change (BirdLife International, 2021b) and, particularly for offshore islands may have been attributed to lack of predators, as is often reported on islets associated with islands with exotic mammalian predators (Barun et al., 2011; Nellis & Everard, 1983; Soorae, 2010).

1.2.5 Habitat

The Grenada Dove inhabits dry, coastal scrub-woodland as well as mixed broadleaf evergreen-deciduous second-growth forests (BirdLife International, 2021b; Rusk, 2017). Such habitat used by the Grenada Dove are areas that were previous sugarcane plantations and agricultural land. Secondary forest that has regrown in these areas appear to be a response to this past land-use (Rusk *et al.* 2008). These forests have dense canopy cover with thorny leguminous trees and shrubs with little ground cover (Rusk, 2017). Dominant tree species in Grenada Dove habitat include: logwood *Haematoxylum campechianum*; gumbo limbo *Bursera simaruba*; black loblolly *Pisonia fragrans*; strongbark *Bourreria succulenta* and wild tamarind *Leucaena leucocephala* (Lugo 2005; Rivera-Milán *et al.* 2015; Rusk 2017). The Grenada Dove is believed to be a habitat specialist and, as such, distribution is closely tied to availability of suitable habitat (Rusk 1998; Rusk *et al.* 2008). While dove density does not appear to be influenced by canopy height or forest type (Rivera-Milán *et al.* 2015), vegetation and canopy cover do appear to be strong environmental determinants for Grenada Dove habitat selection (Bolton *et al.* 2015; Rivera-Milán *et al.* 2015). However, as the Grenada Dove spends a lot of its time on the ground (as discussed in section **1.2.6 Behaviour and Ecology**) it may be that sparse to absent ground-cover

may also be important for this species (BirdLife International, 2021b; Blockstein, 1991). Additionally, the presence of exotic mammalian predators such as the Mona Monkey *Cercopithecus mona* which is present in forest habitat in the centre of Grenada (Rusk, 2009; Zieger et al., 2014), may be a limiting factor as it is often observed that such species are exclusionary to pigeons (Albeshr, 2016; Feeley & Terborgh, 2008; Temple, 1974), hence, the Grenada Dove may be limited to dry lowland forest due to its absence of such predators.

There are differences between habitat on the Southwest and West of the island (BirdLife International, 2021b; Lugo, 2005). Habitat in the Southwest is drier and hotter, with a period of 5-6 dry months dry per year. Mt Hartman is situated in a 1000-1500mm rainfall belt with temperatures ranging between 25-27.5°C (Rusk *et al.* 2008), and elevation and slopes appearing relatively moderate (Lugo 2005; Rusk *et al.* 2008). The surrounding area is composed of residential housing and developments with ongoing construction of new roads, in this area some parts of this habitat extend to the shoreline (Rusk *et al.* 2008; BirdLife International 2021a). Conversely, habitat in the West is moister mixed deciduous forest (Lugo 2005; Rusk *et al.* 2008; BirdLife International 2021a). In some sections of this forest, vegetation type represents a transition between dry forest and humid forest (Rusk *et al.* 2008). There is a higher elevation and steeper slopes in this habitat in comparison to that in the Southwest (Lugo 2005; Rusk *et al.* 2008). Habitat on the West coast is predominately government owned and not heavily used, with evidence of some grazing and cutting in the centre, but otherwise with no other interference to habitat at this site, it is however close to low income housing and adjacent to one of the islands largest landfill sites (Rusk *et al.* 2008; Rusk 2009). Increased information on habitat connectivity and movements of the Grenada Dove will help to identify key areas which may require additional habitat protection and restoration in order to effectively conserve this species. Genetic approaches can identify the level of risk faced by each of these populations and provide information on connectivity between them.

1.2.6 Behaviour and Ecology

Little is known about the behaviour and ecology of this species with a limited number of systematic studies being conducted and a few casual observations made (Blockstein, 1991; Rusk *et al.* 2008; Rusk, 2017). The Grenada Dove is often observed walking on the ground or can be seen perched in trees, remaining in the protective cover of the canopy provided by its habitat (Rusk, 2017). It is rarely observed in flight (Rusk & Clouse, 2004) and when alarmed has been observed flying to the ground to flee on foot (Bolton *et al.* 2015; Rivera-Milán *et al.* 2015). Breeding occurs during the rainy season (June/July–December) and can extend into the dry season in habitat in the West (Rusk, 2017). Little is documented about courtship behaviour of the Grenada Dove (Baptista *et al.* 2020). Grenada Dove build one nest, around 4m above the ground, which is a fragile platform of sticks lined with rootlets (Blockstein 1991; Baptista *et al.* 2020). Vocal behaviour is associated with breeding with calling, heard primarily during the breeding season, being used to mark territory and for mate attraction (Blockstein, 1991). Vocalisation is heard from dawn to late morning, rarely around midday (the hottest part of the day) and again in late afternoon (Rivera-Milán *et al.* 2015). Calling occurs from branches around 4-6m above ground (Blockstein & Hardy, 1989). Nests can be located using Grenada Dove vocalisations and a number of observations have been made when monitoring nests, for instance: a) when eggs are present, the nest is never left unattended, with males and females switching to achieve this, often with one dove on the nest and one close by, usually on the ground; b) more movement has been observed by adults on the nest when eggs are present; c) nest are re-used; and d) when approaching an occupied nest the male will usually fly to the ground and walk away which is thought to act as distraction from the nest (personal communication with Forest Ranger Doland Francis in May 2017).

The Grenada Dove appears to be a frugivorous, insectivorous and granivorous ground-feeding species (Twyman and Hayslette 2007; Baptista *et al.* 2020). Although the species' precise diet (and the diet of many other *Leptotila* species) remains largely unknown, it is thought to feed primarily on seeds of *Leucaena leucocephala*, *Bourreria succulenta*, *Faramea occidentalis*, *Bauhinia aculeata* and

Haematoxylum campechianum (Twyman and Hayslette 2007; Bolton *et al.* 2015; Rusk 2017; BirdLife International 2021a). It is also known to feed on small fruits from fruit-baring trees such as genera *Forrestieria, Exostema, Bourreria, Coccoloba, Chomelia, Randia, Leucaena, Citharexylum* and *Bursera* present in Grenada Dove habitat (Lugo, 2005; Twyman & Hayslette, 2007). Insect consumption includes mealworms (in captivity), snails, grubs and caterpillars (Twyman and Hayslette 2007; Baptista *et al.* 2020). The Grenada Dove forages exclusively on the ground and while predominately observed foraging alone it has occasionally been observed foraging in pairs (Blockstein 1988; Rusk 1998; Rusk and Clouse 2004; Baptista *et al.* 2020). Although the Grenada Dove has been observed foraging close to clearing edges it has not been observed foraging outside of forest habitat (Baptista *et al.* 2020).

1.2.7 Threats to the Grenada Dove

Habitat loss and degradation are perhaps the main threats to the already limited range of this species (Rivera-Milán *et al.* 2015; Rusk 2017; BirdLife International 2021a). Initially, dry forest was cleared for small plantations, charcoal production and other industrial developments, however in more recent years it has been cleared for the building of residential housing, roads and commercial developments (BirdLife International, 2021b; BirdsCaribbean, 2020a). One of the more recent potential commercial developments is a proposed tourist resort and golf course at Mt Hartman Estate and surrounding area; arguably the most important habitat for this species and home to the largest population of Grenada Dove (Rusk 2008; Rusk *et al.* 2010; BirdsCaribbean 2020a). In addition to commercial development, habitat in the Southwest is at risk of fly tipping (Figure 1.4a.), human activity and tethered livestock (Rusk *et al.* 2008; Bolton *et al.* 2015; Rivera-Milán *et al.* 2015). Habitat in the West is separated by one road from Grenada's main waste processing plant (Figure 1.4c. and 1.4d.) and is consequently not only at risk from pollution, toxic fumes, fire, overspill and fly tipping (Figure 1.4b.), but also the potential expansion of the landfill site itself (Rusk *et al.* 2008; Rusk 2009; BirdLife International 2021a).

While Grenada lies just south of the hurricane belt, the island has been hit by a number of hurricanes which have had catastrophic effects on the island's ecosystems, agricultural sector and

economy (Rusk, 2009). The risk of hurricanes is predicted to increase with climate change (Nelson *et al.* 2018). As well as destruction to Grenada Dove habitat, a change in forest structure was observed after hurricane Ivan in 2004 (Rusk *et al.* 2008), as the hurricane felled trees and upturned roots, altered canopy cover, introduced debris into forest areas and instigated the invasion of alien vegetation such as vines (Rusk *et al.* 2008; BirdLife International 2021a). All of which affected the ability of the dove to move through the habitat. A lower number of calling males were observed after the hurricane, possibly due to loss of life, but also potentially due to stress induced from the limited resources of a post-hurricane landscape (Rusk, 2005, 2017).

Figure 1.4: a. Photo of fly tipping at Mt Hartman (2019); b. Photo of fly tipping at Perseverance (2019); c. and d. photos of the landfill site adjacent to Perseverance Dove Sanctuary (2017). All photos taken by the author.

Introduced predators pose a substantial risk to the Grenada Dove population, and while the literature on the Grenada Dove appears to go back to the 1800's when it was first described (Lawrence, 1884; Wells, 1886) a number of exotic mammalian predators would have already been introduced and

become well established by this time (Zieger et al., 2014).The direct impact of predation on this species is yet to be quantified and there are a number of potential risk factors (Rusk et al., 2008). Invasive alien species are known to be a major cause of biodiversity loss leading not only to disturbance and predation of native species but also increased competition, modification of habitats and ecosystems, enhanced disease transmission, risk of genetic integrity via hybridisation and increased brood parasitism (Lees et al., 2022). Not only is it thought that predators eat the eggs and fledglings of this species (Rusk 2017), the Grenada Dove spends a lot of time foraging on the forest floor thus increasing its vulnerability to predators (Bolton *et al.* 2015). Mammals which have been introduced to the dry forest in Grenada include Feral Cats *Felis catus*, two species of Rats *Rattus rattus* and *Rattus norvegicus*, and Manicous *Didelphis marsupialis*, with Mongoose *Herpestes auropunctatus* in particular being prevalent in Grenada Dove habitat (BirdLife International, 2021b; Twyman & Hayslette, 2007). The Small Asian Mongoose is native to South and Southeast Asia and was introduced to Grenada in the 1870s as pest-control measure for Rats and Cats on sugar cane plantations. However, Mongooses were ineffective as pest control and subsequently established their own viable populations on the island. The Mongoose population on Grenada is estimated at around 200,000 and averaging at around six per hectare (Zieger *et al.* 2014). Other exotic mammalian predators present on Grenada include the Mona Monkey *Cercopithecus mona* (Rusk, 2009; Zieger et al., 2014), and although it does not inhabit the dry forest, may be limiting factor which alters the distribution and habitat preference of the dove (BirdLife International, 2021b; Lees et al., 2022).

Freshwater sources are limited in all Grenada Dove habitat and the increasing risk of climate change, with resulting drying climatic trends, is further limiting the availability of freshwater (Rusk 2017; BirdLife International 2022b). Unlike habitat in the West which does have some freshwater sources, such as Beausejour river and Black Bay river, habitat in the Southwest has little-to-no permanent watercourses (Forteau 2010; Rusk 2010, 2011; Bolton *et al.* 2015). Water sources at Mt Hartman are comprised of standing water sources in the form of nine man-made wells (Figure 1.5) and watering holes (Rusk 2011; Bolton *et al.* 2015). These wells are dependent on rainfall to fill them,

which is scarce during the dry season between the months of January and May/June (Rusk, 2011, 2017). Research conducted by Bolton *et al.* (2015) obtained camera trap footage of the Grenada Dove drinking at man-made wells within Mt Hartman estate alongside other columbids as well as predators. Despite evidence that Grenada Dove are dependent on these water sources (Bolton *et al.* 2015), it is possible that these wells will be removed if hotel development plans are approved for this site (Rusk, 2011).

Figure 1.5: Example of a standing water source at Mt Hartman Estate. Photo taken in April 2017 by the author.

Standing water, especially when shared with other columbids, can aid and increase the spread of diseases such as *Trichomoniasis*, which primarily affects Columbiformes (Bunbury *et al.* 2008). *Trichomonas gallinae*, is a flagellate protozoan which is known to cause high mortality in squabs and can be transmitted between columbid species (Ewen, 2012). Mosquito-carried avian malaria may also pose a threat to Grenada Dove populations (Fallon *et al.* 2005; Bolton *et al.* 2015). Although it is not

yet known what diseases are prevalent in Grenada Dove habitat or what effect they are having on this species, the introduction of exotic organisms and the subsequent spread of pathogens and avian disease is a known extinction risk for endemic island species (Santiago-Alarcon *et al.* 2006).

Lack of knowledge in regards to fundamental biological and ecological information for the Grenada Dove includes: survival and mortality rates; home-range size; life history traits; sex ratio, predator effects; habitat use and preference; genetic diversity; disease prevalence and behavioural patterns – all of which are limiting factors in the conservation of this species (BirdLife International, 2021b; Bolton *et al.* 2015; Rusk, 2017).

1.2.8 Grenada Dove Conservation

There are a variety of conservation actions underway to help protect the Critically Endangered Grenada Dove from extinction. These actions are predominately being conducted by researchers associated with the Grenada Dove Conservation Programme and the Forestry and National Parks Department of the Government of Grenada, along with regional and international collaborators (BirdLife International, 2021b; Rusk, 2014). The Grenada Dove has legal protection from hunting and egg collection, however, this is not common practise for this particular species in Grenada and does not constitute a particularly large threat (BirdLife International, 2021b). Additional legal protection is provided to protect habitat at two locations, Mt Hartman National Park and Perseverance protected Area and Dove Sanctuary as mentioned previously in section **1.2.4 Range and Historical Distribution** (Bolton *et al.* 2015; Rusk, 2017). Grenada Dove habitat is managed by the Forestry and National Parks Department and includes patrolling by Forest Rangers, maintenance of fences and boundaries, signage (Figure 1.6) and rubbish clearing (BirdLife International, 2021b; Forteau, 2010; Lugo, 2005). This also encompasses a predator-control programme whereby traps (Figure 1.7) are distributed throughout Grenada Dove habitat to trap non-invasive predators for removal (Rusk, 2017). Forest habitat at Mt Hartman has to date been successfully defended from proposed commercial development though the use of Environmental Impact Assessments (EIAs), along with consultations between conservationists and developers to find a compromise that ensures the survival of the Grenada Dove if development is

to proceed (Rusk 2010). The current proposed development (2022) is one in a number of times since the 2000s that planning permission has been sought to build at habitat in the Southwest region of the island (Rosenberg & Korsmo, 2001; Rosenberg, 2018).

Additional conservation support for the Grenada Dove includes increasing awareness of this Critically Endangered endemic species, such as the assignment as the national bird of Grenada. Consequently the Grenada Dove features on the countries stamps and is the focus of education (Figures 1.8 and 1.9) and ecotourism programmes (BirdLife International, 2021b). Furthermore, collaborations with regional and international researchers have contributed to an increase in knowledge of population size, threats, behaviour and habitat for this species (Blockstein & Hardy, 1989; Bolton *et al.* 2015; Devenish-Nelson & Nelson, 2021; Nelson *et al.* 2018; Rivera-Milán *et al.* 2015; Rusk, 2017; Twyman & Hayslette, 2007).

Figure 1.6: Signage use in Grenada Dove habitat at a) and b) Mt Hartman and c) Perseverance. Photos taken by the author in 2019.

Figure 1.7: a. Example of an invasive predator trap used at Mt Hartman (2019) and b. a small Asian Mongoose caught in a trap (2017). Photos taken by the author.

Figure 1.8: Examples of eduational information available at Mt Hartman National Park a) Mt Hartman visitor centre (photo by Ellie Devenish-Nelson, 2017), b) information board inside the visitor centre (2017) c) information boards outside the visitor centre (2019) and d) information board showing the Grenada Dove (bottom, centre left). Photos taken by the author unless otherwise stated.

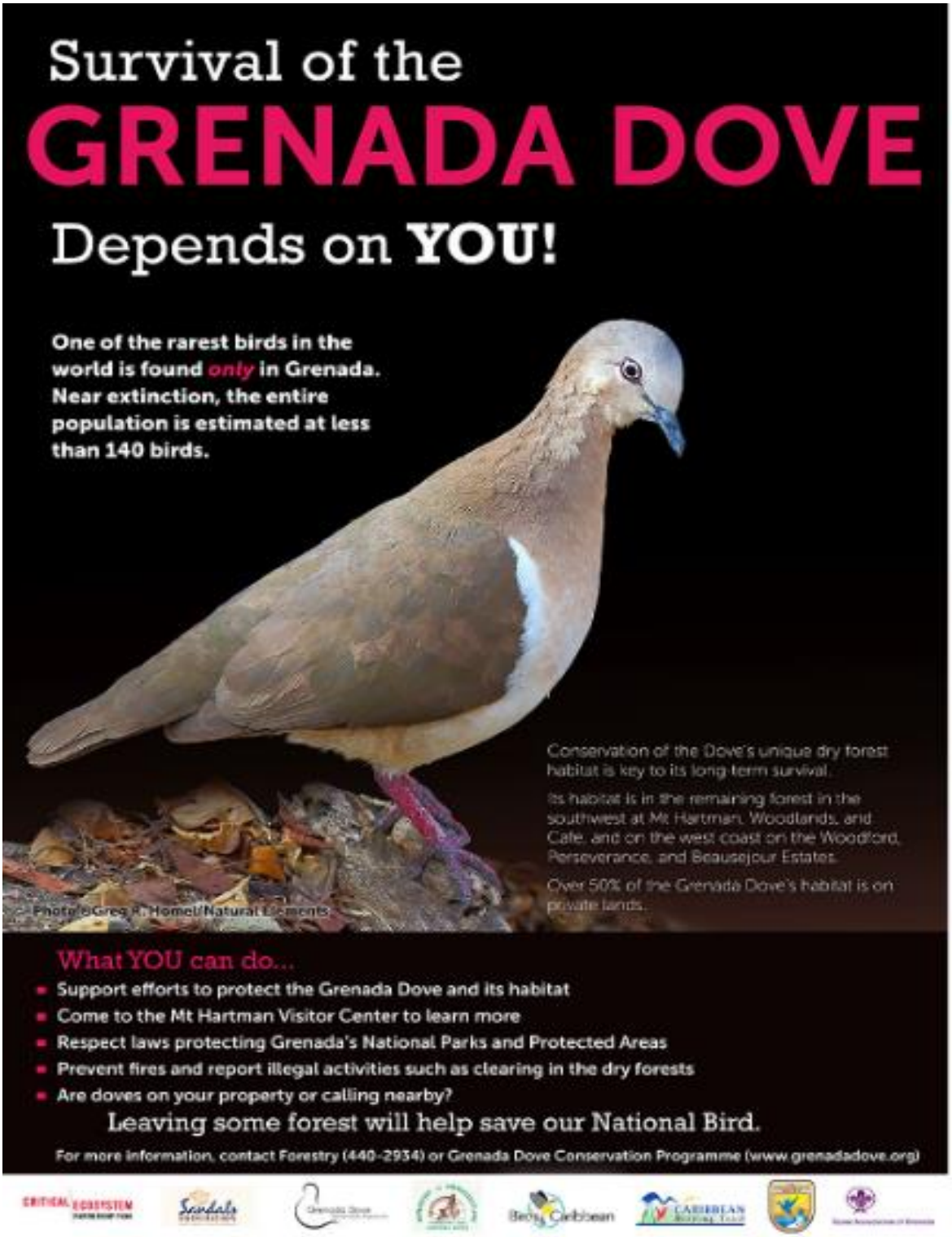

Figure 1.9: Poster produced for education and awareness programmes obtained from:(BirdsCaribbean, 2020b)

Further proposed conservation actions are outlined in the recovery and action plan for the Grenada Dove (Rusk *et al.* 2008). This includes additional habitat protection and restoration, minimising further habitat loss, providing alternatives to standing water sources (i.e. leaky hose pipes to reduce impacts of disease), establishing long-term monitoring programmes for both doves and habitat, expanding the predator control programme and conducting comprehensive ecological research (BirdLife International, 2021b, 2022b; Rusk *et al.* 2008; Rusk, 2017). Consideration of a captive breeding programme and establishment of new populations is also proposed in the species recovery plan (BirdLife International, 2021b, 2022b; Rusk *et al.* 2008). Future plans will continue to provide opposition to developments at Mt Hartman and the surrounding area (BirdLife International, 2022b). Additional recommendations include banding programmes to obtain outstanding information (home range size, sex ratio, life-history parameters, genetic diversity) and remote camera surveillance to monitor nesting success and failure rates, along with research into the Grenada Dove's diet and subsequent planting programmes to increase habitat resource availability (Bolton *et al.* 2015).

1.2.8 Grenada Dove genetics

Genetic investigation of the Grenada Dove has not previously been conducted. Consequently, information regarding phylogenetic placement, evolutionary history, population connectivity and genetic diversity are outstanding. Genetic evaluation will contribute to increasing fundamental biological knowledge for this species.

1.3 Aims and objectives of the PhD

This project aimed to answer the following question:

What is the genetic status of the Critically Endangered Grenada Dove *Leptotila wellsi* and what conservation management recommendations can be made which will help to ensure the long-term survival of this species?

In order to answer this question, I aimed to complete the following objectives:

1) Review genetic studies exploring the conservation of island birds.

2) Develop the usability of non-invasive samples as a source for informative genetic material for bird conservation.

3) Assign the phylogenetic placement of the Grenada Dove within Neotropical Columbids.

4) Investigate the mitochondrial genetic diversity of two Grenada Dove populations.

5) Predict and compare the impacts of threat and management scenarios on the long-term survival of the Grenada Dove.

In order to obtain genetic information for the Grenada Dove, opportunistically collected feather and egg shell samples were provided by the Forestry and National Parks Department of the Government of Grenada. As this species is listed by CITES (Convention on the International Trade in Endangered Species) samples were imported into the UK using CITES import (546012/01, 567389/01) and export licences (Grenada IACUC 14002, 14004). This project will provide the first genetic analysis of the Grenada Dove and reveal its phylogenetic placement among other Neotropical Columbid species, it will also use phylogenetic comparative methods to predict previously unreported life-history traits and investigate genetic diversity at a population level. Using PVA methods to assess the projected effects of threats and management of this Critically Endangered species, this project will identify the greatest risk factors and most effective management strategies in order to discuss conservation management and the long-term viability of Grenada Dove populations.

I present my methods, analyses and results in the following chapters:

Chapter 2: Geographic bias and under-representation of threatened species in conservations genetics: A systematic literature review of island bird genetics

Overview: Literature on the genetics of island birds is exported and reviewed. Information regarding the geographic location, methodologies used, taxa studied and conservation recommendations are evaluated, and trends identified. The findings of this review and any biases identified are discussed, along with the possible limitations of the application of genetic research to conservation.

Chapter 3: A novel method to optimise the utility of underused moulted plumulaceous feather samples for genetic analysis in bird conservation

Overview: Alterations to a user-developed protocol are made, along with the use of a primerless PCR technique and small amplicon strategy to obtain informative genetic material from opportunistically collected feather and egg shell samples. The use of non-invasive sampling for Critically Endangered species is discussed.

Chapter 4: Phylogenetic placement and life history trait imputation for Grenada Dove *Leptotila wellsi*

Overview: The phylogenetic placement of the Grenada Dove is revealed using data from three mitochondrial markers and one nuclear marker, and the evolutionary history of this species is discussed. Phylogenetic comparative methods are used to predict previously undocumented life history traits, and comparisons to other closely related Neotropical columids are discussed here.

Chapter 5: Mitochondrial genetic analysis of the Critically Endangered Grenada Dove *Leptotila wellsi* reveal isolated populations with low mtDNA diversity

Overview: Low mitochondrial genetic diversity is observed using data from two genetic markers. Two haplotypes are identified, and isolation of the two extant populations of the Grenada Dove is revealed. Molecular sexing indicates a male-sex bias in the samples. The importance of incorporating genetic measures in conservation management for isolated Grenada Dove populations is discussed.

Chapter 6: Assessing threats and conservation action using Population Viability Analysis for the Critically Endangered Grenada Dove *Leptotila wellsi*

Overview: Population viability analysis is performed for the Grenada Dove using open-source software VORTEX version 10. This PVA is used to assess three scenarios of potential threats to the survival of this species. Three conservation management scenarios are modelled to predict their effect on population growth and viability. The effect of different threats and types conservation management

on the population trajectory, along with the most harmful and beneficial scenarios for the Grenada Dove are discussed.

Chapter 7: Discussion and future research

Overview: The implications of the findings of this study are discussed, along with the potential for further genetic investigation for this Critically Endangered species. Additions and alternations to the species recovery programme in light of the genetic findings of this study are discussed along with future population projections of the Grenada Dove.

CHAPTER 2 SYSTEMATIC REVIEW

Photo by Author

Chapter 2: Geographic bias and under representation of threatened species in conservation genetics: A systematic literature review of island birds

2.1 Abstract

Island birds are at an increased risk of genetic isolation, inbreeding depression and reduced evolutionary potential. As such, the integration of genetic rescue measures into conservation management is an important strategy in island bird conservation. The aim of this study was to investigate how genetic approaches have been applied to the conservation of island bird species. To do this, I conducted a systematic review of literature about conservation genetics of island birds by searching Web of Science (WoS) and Bielefeld Academic Search Engine (BASE) databases. A final number of n=535 studies were then reviewed. An increase in genetic studies was observed over time. The Oceanic realm was by far the most studied geographical area (54% of studies) with the majority of studies based in New Zealand. The most studied order of birds was Passeriformes (35% of studies), however, no species was studied particularly more than any other. Overall 55% of studies made conservation recommendations but the majority of studies were conducted on non-threatened species (69% of studies). The findings of this study reveal geographical bias, under-representation of threatened species and highlights the gap between genetic research and active conservation management. It is recommended that future studies focus on under-represented areas whilst prioritising threatened species to carry out genetic research which directly informs conservation.

2.2 Introduction

Occupying just 5.3% of global land area, islands host more than 20% of the world's vertebrate and plant species (Courchamp *et al.* 2014; Tershy *et al.* 2015). Islands experience unique evolutionary processes and natural-selection pressures due to their isolation, some with a relatively young age, limited spatial extent and closed boundaries; as such island populations are ideally positioned for providing insights to adaptive radiation and speciation (Losos and Ricklefs 2009; Copsey *et al.* 2018). The distinct evolutionary history of islands mean they are hotspots of biodiversity and endemism (Kier *et al.* 2009; Courchamp *et al.* 2014; Spatz *et al.* 2017). Such hotspots include the islands of South-East Asia and the Caribbean, along with African and Hawaiian islands, all of which show extremely high levels of endemism (Goodman and Benstead 2005; Kier *et al.* 2009; Gillespie *et al.* 2013; Hughes 2017; Devenish-Nelson *et al.* 2019). Insular systems are also hotspots of extinction (Courchamp *et al.*2014; Spatz *et al.* 2017) as island ecosystems are more vulnerable to invasive alien species, habitat fragmentation, climate change, human population growth and land use changes compared to mainland ecosystems (Gross 2006; Courchamp *et al.* 2014; Yamano *et al.* 2015; Pruett *et al.* 2017; Copsey *et al.* 2018). Consequently, 61% of extinct animal species listed by the IUCN Red List of Threatened Species are island restricted (Tershy *et al.*2015). Despite evidence that island conservation is of critical importance to the survival of many species (Donald *et al.* 2010) there remains geographic bias towards typically mainland areas, with temperate zones and wealthy countries over-represented in the literature (Vamosi and Vamosi 2008; Martin *et al.* 2012). Although, avian species richness is not evenly distributed across biogeographic realms - with higher levels of biodiversity observed in the tropics for example - it has been noted that scientific focus and funding does not always align with these hotspot regions of high conservation priority (Martin *et al.* 2012; Belle *et al.* 2019; Lees *et al.* 2022).

Although birds are globally widespread and present on every continent (Jenkins *et al.* 2013), almost a fifth of all bird species exist on islands (Simberloff 1999; Tershy *et al.* 2015). Over 150 bird species have become extinct in the last 500 years, with more than 90% confined to islands (Simberloff

1999; Donald *et al.* 2010; Szabo *et al.* 2012). While island birds face much of the same threats as continental species, their often small population sizes and limited ranges increase their vulnerability to a variety of threats; in particular hunting, natural disasters, disease and the impact of invasive species (Donald *et al.* 2010). As a result , island birds are considered to be of critical concern with 58.8% of extant Critically Endangered avifauna are insular species (Tershy *et al.* 2015).

Birds are distinct from other taxa, as their ability to fly often means they are not restricted by geographical barriers in the same way as other land bound taxa (Grinnell 1914; Butler 2016; Kozakiewicz *et al.* 2018). This, along with unique avian life history traits, breeding behaviours and ecology, has an effect on the genetic variation observed in bird populations (Maan and Seehausen 2011; Kozakiewicz *et al.* 2018). Despite their ability to fly island bird populations often have a low dispersal capacity (Moore et al., 2008) and are prone to low genetic variation (Hudson *et al.* 2000), inbreeding depression (Swinnerton *et al.* 2004), genetic isolation (Jouventin *et al.* 2006) and reduced evolutionary potential (Lovette *et al.* 1998). They are also at risk of introgression (Lawson *et al.* 2017), reduced heterozygotic fitness (Monceau *et al.* 2013b) and genetic drift (Miller & Lambert, 2004). Natural genetic rescue is unlikely in many island populations due to lower genetic diversity, limited dispersal ability and small declining populations (Pruett *et al.* 2017); therefore, integrating genetic rescue measures into conservation management is an important strategy in island bird conservation (Heber *et al.* 2012). However, disproportionate attention is often paid towards charismatic and flagship species leading to a misalignment between species studied and those most in need of genetic rescue and management strategies (Lawler *et al.* 2006; Ducarme *et al.* 2013; Di Marco *et al.* 2017; Kozakiewicz *et al.* 2018).

The pressing issues of climate change and habitat loss mean that island populations are becoming ever more fragmented, reducing population size and gene flow (Weeks *et al.* 2011). As such genetic management of small isolated populations is becoming increasingly important for insular species (Frankham 1997; Frankham *et al.* 2019). Molecular methods used are often dependant on the species and sample type. For example, it may be difficult to obtain permits and permission to

invasively sample some species; likewise, elusive and dangerous species may be difficult to trap meaning non-invasive samples or environmental DNA (eDNA) are relied upon (Vili *et al.* 2013; Peters *et al.* 2019). Other limitations, including availability of resources and funding, will determine the type of genetic data which can be generated with newer technologies, such as next-generation sequencing (NGS) and whole genome scale analysis, being more expensive as well as requiring specific training (Hoban *et al.* 2013b; Kress *et al.* 2015; Mable 2019). This will in turn have an impact on the type of research conducted as different genetic markers and methodologies are used to address different conservation issues, therefore influencing the resulting measures and conclusions made for conservation (Wan *et al.* 2004; Hoban *et al.* 2013a, b).

A large variety of genetic measures such as genetic augmentation, developing captive-breeding programmes and creating new populations can be actioned by conservationists in species recovery plans (Taylor and Jamieson 2008; Weeks *et al.* 2011; Bowker-Wright *et al.* 2012). Establishing gene flow to increase population connectivity and minimising breeding between related individuals for contemporary populations can help reduce the genetic risks to island populations (Ramírez *et al.* 2013; Frankham *et al.* 2019; Ralls *et al.* 2020). The inclusion of historical samples in genetic research can provide information about the demographic history, pinpointing loss of genetic variation indicative of population bottlenecks (Feinstein *et al.* 2008; Sánchez-González *et al.* 2015; Copsey *et al.* 2018). For instance, Gregory *et al.* (2012) used microsatellite markers to assess the genetic structure of a newly established island population, allowing translocation of outbred individuals and thus reducing inbreeding and its deleterious effects in the new population. Such studies highlight the importance of genetic approaches and how they can positively influence conservation efforts (Gregory *et al.* 2012). Despite the documented benefits of the utility of genetic information for conservation a gap has emerged between genetic research and its real-world application to conservation management (Hoban *et al.* 2013b; Britt *et al.* 2018). Consequently, the large number of studies investigating the genetic composition of island birds do not all translate to conservation action

contributing to the preservation of the species they examine (Kahilainen *et al.* 2014; Taylor *et al.* 2017).

Here I investigated how genetic approaches have been applied to the conservation of island bird species. In particular, I answered the following questions: 1) is there a trend in the types of molecular methods used?; 2) are there some island groups which are more studied than others?; 3) which species are the focus of genetic studies?; and 4) do these studies make specific conservation management recommendations? I performed a systematic literature search and categorised key information pertaining to my research questions from each study to identify trends in the literature. I used this information firstly to identify island groups and species currently understudied that should be the focus of further genetic research; secondly to evaluate the utility of sampling and molecular methods for future studies; and thirdly to make recommendations on how to increase the application of genetics in active conservation management.

2.3 Methods

I conducted a systematic review of the literature around the conservation genetics of island birds by searching Web of Science (WoS) and Bielefeld Academic Search Engine (BASE) databases. The WoS 'All Databases' search option was used (including the Web of Science Core Collection, BIOSIS Citation Index, Data Citation Index, KCI-Korean Journal Database, MEDLINE®, Russian Science Citation Index, SciELO Citation Index under the University of Chester's institutional subscription). BASE was searched in order to include any additional resources such as institutional repositories and "Deep Web" - content not indexed by web databases or search engines (Essien, 2019) - resources which may not be captured by WoS.

I used the automated approach outlined by Grames *et al.* (2019) to identify search terms based on keyword co-occurrence networks using the R package litsearchr in R version 4.0.0 (R Core Team, 2021). I conducted an initial search between the 20th and 23rd July 2020 using the following terms in order to retrieve studies about birds (bird* OR avian OR ave*) AND genetics ('conservation

genetic*' OR 'landscape genetic*' OR 'population genetic*' OR phyloge*). I used advance search options set to include English language studies and research paper, data paper and postgraduate thesis document types (Appendix I: Table 1). The search results were sorted by relevance and the first 300 from each search engine were imported into R. Litsearchr was then used to remove duplicates, identify potential keywords, build the keyword co-occurrence network and identify change points in keyword importance to generate a list of possible keywords. These automated keywords were reviewed manually and were grouped into the following concept categories: bird, genetic, conservation, range and NOT (exclusion of specific search terms) to determine which terms were to be included. A search string of initial search terms and terms in concept groups were used to write Boolean searches and generate a list of search terms appropriate for use in the final search.

Upon investigation of the automated keywords, I conducted the final search on the 14th August 2020 using the following search terms from each concept category: (bird* OR avian OR aves) AND ('landscape genetic*' OR phylogenetic OR phylogeography OR 'molecular genetic' OR 'avian genom*' OR 'population genetic') AND ('conservation genetic' OR 'conservation genomic' OR conservation) AND ('island species' OR 'island population' OR endemic OR 'island endemic') NOT ('avian malaria' OR 'plasmodium relictum' OR influenza OR parasit* OR vector) with the same advance search options set above. I checked these results against a list of 10 "gold standard" relevant articles, as per the method outlined by Grames *et al.* 2019, which were on the topic of island bird genetics to test the search strategy for precision and recall (see Appendix I: Table 2). The resulting number of articles after the removal of duplicates was n=1921.

The final exported articles were then manually filtered using the title and abstract and any studies that were not quantitative and descriptive data studies about the genetics of island bird populations (see Appendix I: Table 3 for full list of criteria) were removed leaving a final number of n=535 to be reviewed. A database of the remaining articles was constructed including title, author, year, publisher/source and document type for each article. Each article was reviewed and the following information was extracted from each study: 1) Which molecular markers and methods were

used?; 2) Which geographical realm (BirdLife International, 2022b) and specific islands within them were the focus of the research?; 3) What species were studied?; and 4) Did the research make conservation recommendations? A comprehensive list of review categorisation criteria can be found in Supplementary Material 1: Table 4. To reduce complexity of categorisation - molecular makers were grouped as follows: mitochondrial DNA markers, specific functional genes, sexing markers and all other nuclear DNA markers (see Appendix I: Table 4 for more detail) - due to the convoluted nature of methodologies reported. Where information wasn't provided for any particular category no data was recorded for that specific section.

Analysis for island populations of birds (i.e. bird populations on islands including island populations and continental populations comparisons and populations of island breeding birds) was performed on all (n=535) articles. Of these (n=296) were focused solely on island restricted species (i.e. birds with no existing continental populations).

2.4 Results

2.4.1 Methodological trends

There was an increase in molecular studies over time for all marker types (Figure 2.1) which showed the sharpest increase in both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) markers (as categorised by this study), with 61% and 64% of studies using these marker types respectively, showing no substantial difference between the two. Over a third of studies (39%) used more than one marker type. Molecular sexing was used in around 13% of studies and functional genes, which were not presented until the 2010's, were the least studied marker type, used in around 3% of studies.

The majority of samples in the reviewed studies were collected invasively (57%) and the largest proportion of these (88%) were from wild/contemporary populations (Figure 2.2). Non-invasive sampling was the least used method for obtaining samples (11%) and the largest proportion of these (78%) were museum/ancient samples. A substantial proportion of studies (23%) used a

combination of both invasive and non-invasive techniques in order to obtain samples. There was no evidence that sampling method was related to species conservation status (Figure 2.3).

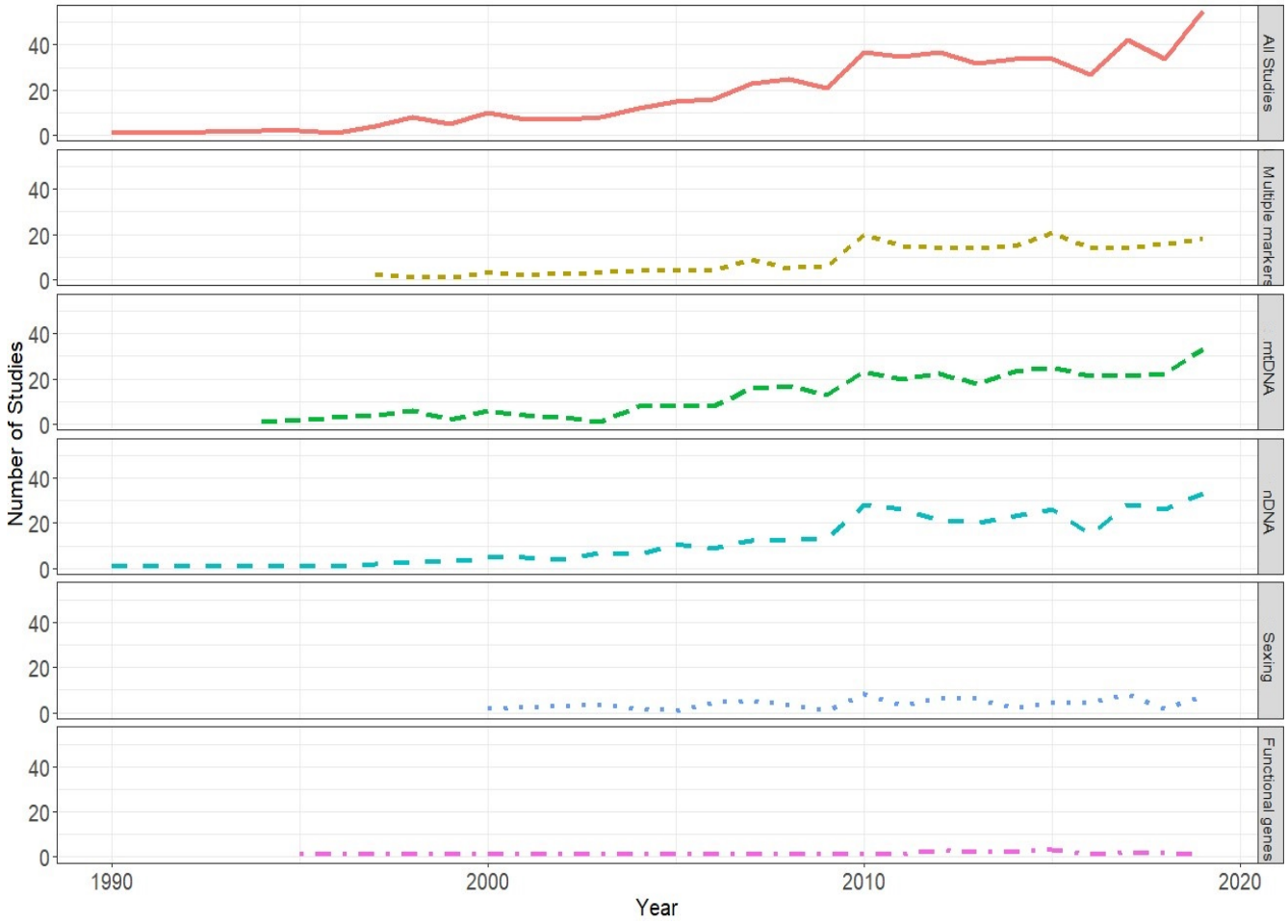

Figure 2.1: Number of conservation genetic studies published per year (1990-2020*) for each molecular marker *As the literature export was carried out in June 2020 there was limited data for the year 2020 as such the numbers for 2019 include the first 6 months of 2020.

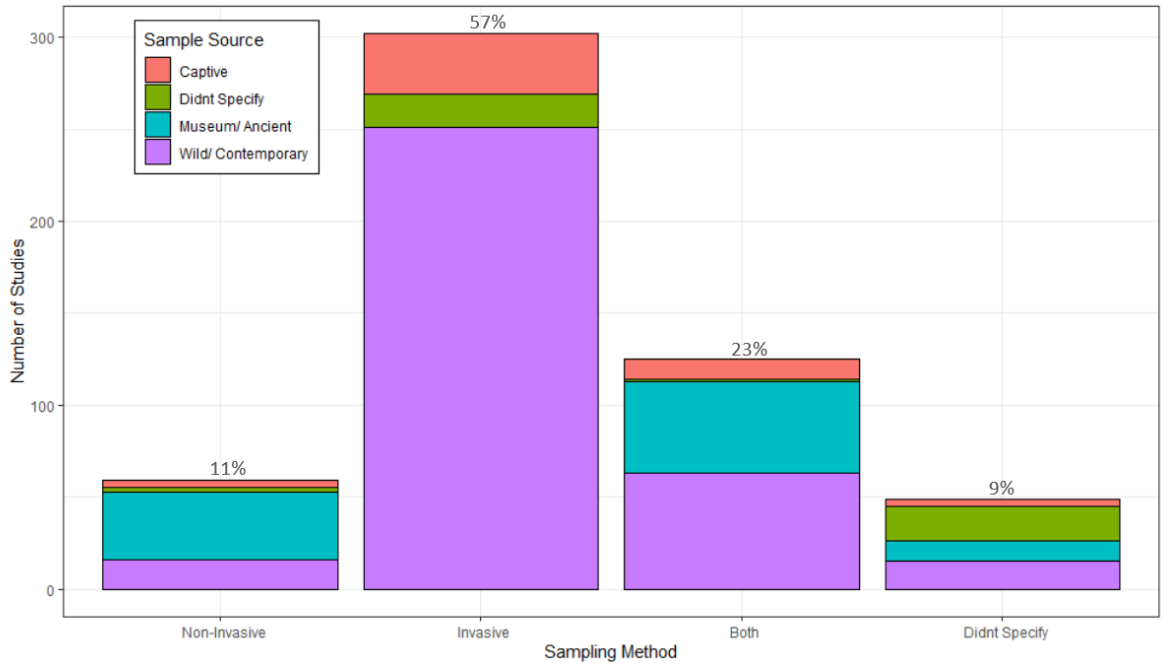

Figure 2.2: Total number of studies per sampling method and the proportion of samples which were from Wild/Contemporary, Museum/Ancient or Captive sources.

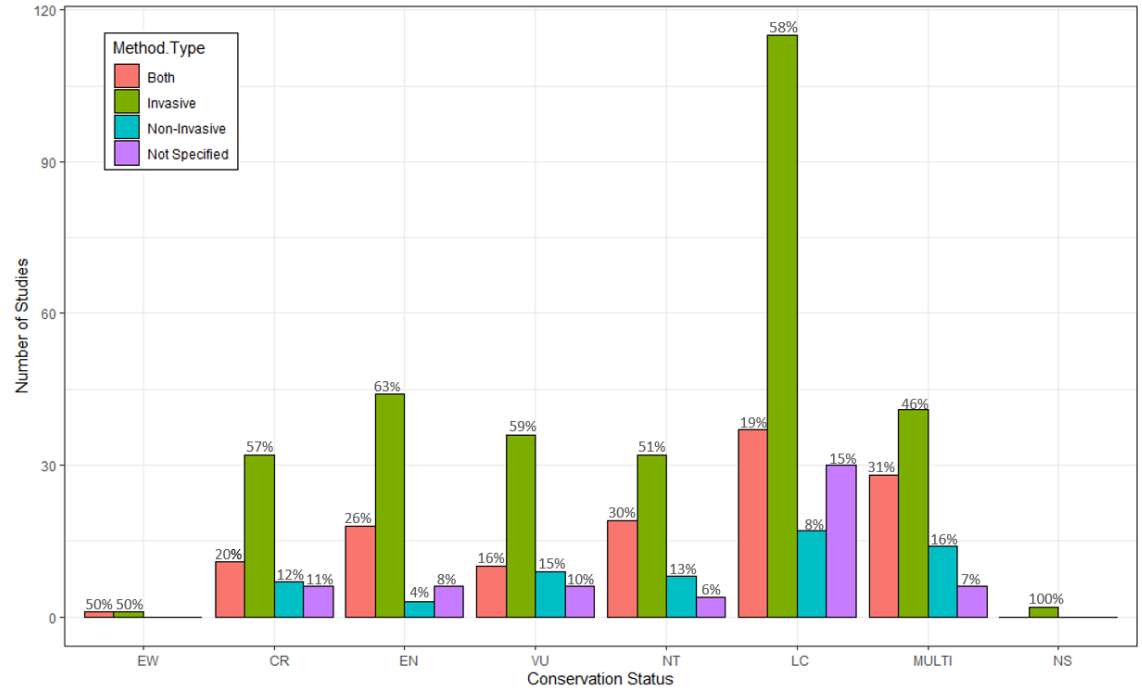

Figure 2.3: Comparison between the numbers of published studies for each Conservation Status and the numbers which used each sampling method. Extinct in the Wild (EW), Critically Endangered (EN), Endangered (EN), Vulnerable (VU), Near Threatened (NT), Least Concern (LC), Not Specified (NS)

2.4.2 Geographic distribution

The Oceanic realm was by far the most studied area (Figure 2.4) with the large majority of studies based on New Zealand (54% of studies in the Oceanic realm) and the Hawaiian Islands (15%). New Zealand was the most studied group of islands globally. The Antarctic realm was studied the least. When considering only island restricted species, realms in the tropics and subtropics (Afrotropical, Indomalayan and Neotropical) were the most studied after the Oceanic realm with notably fewer studies within realms in the temperate and frigid zones (Antarctic, Nearctic and Palearctic). A large proportion of each geographical realm was represented by a few islands. The Afrotropical realm was largely comprised of studies conducted on the Seychelles (26%), the Canary Islands (13%) and Madagascar (15%). The remaining realms had one key set of islands which were the focus of a number of studies in that location which were as follows Indomalayan – Philippines (30%), Neotropical – the Galápagos Islands (49%) and Palearctic – Japanese Islands (54%).

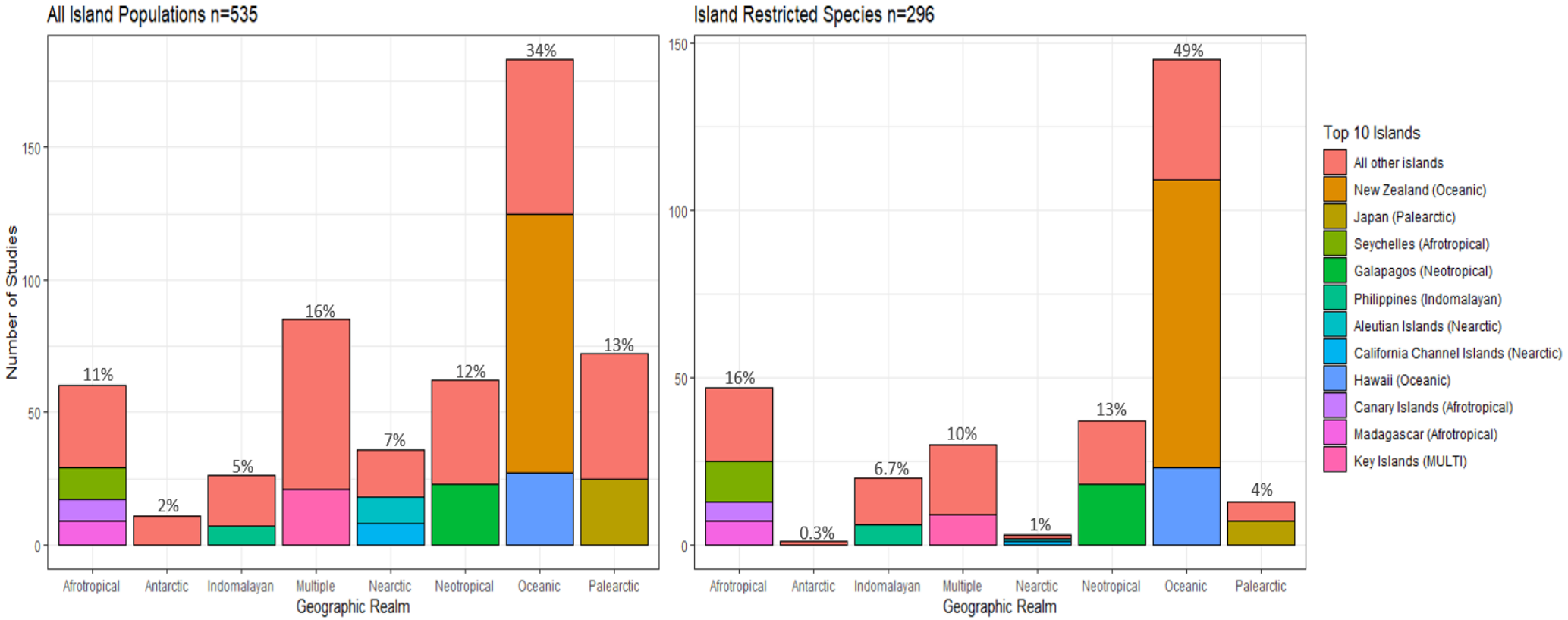

Figure 2.4: Geographical distribution of conservation genetics studies showing the proportion of the top ten studied island groups per geographical realm.

2.4.3 Taxonomic patterns

The most studied orders were Passeriformes (35% of studies), Procellariiformes (13%) and Charadriiformes (12%; Table 2.1). For Passeriformes this was spread over 48 families, 85 genera and 102 species. The top three most studied family groups were Procellariidae, Phasianidae, Anatidae and the most studied genera were *Pterodroma*, *Petrocia* and *Acrocephalus*. When considering single species studies of island populations, the most studied birds were *Melospiza melodia* (6 studies), *Petroica australis* (6 studies), *Strigops habroptila* (6 studies). When considering island restricted species, the top three most studied orders were Passeriformes, Psitiaciformes and Procellariiformes respectively. The most studied family groups were Procellariidae, Anatidae and Psittaculidae respectively. The top three studied genera were the same as above but with *Petrocia* being the highest. When considering single species studies of island restricted birds, the most studied species were still *Petrocia australis* and *Strigops habroptila* with the addition of *Notiomystis cincta* (5 studies) in place of *Melospiza melodia* which is not island restricted. Proportion tests show that the number of families (χ^2= 38.212, df = 24, p-value < 0.05), genera (χ^2= 207.19, df = 24, p-value < 0.05) and species (χ^2= 593.39, df = 21, p-value < 0.05) studied were significantly lower than the total number in the corresponding group. Only in the following orders were all families (Apterygiformes, Ciconiiformes, Columbiformes, Falconiformes, Mesitornithiformes, Otidiformes, Phaethontiformes, Podicipediformes, Psittaciformes, Sphenisciforme, Strigiformes) and all genera (Apterygiformes and Phaethontiformes) represented.

2.4.4 Conservation recommendation trends

Overall 55% of studies made conservation recommendations which is reduced slightly to 54% when examining only island restricted species (Figure 2.5). Furthermore, approximately 28% of studies make taxonomic recommendations and 22% for island restricted species. Studies on multiple taxa and Least Concern species have higher number of studies making taxonomic recommendations. Proportionately, studies on threatened species (conservation status: VU, EN, CR) were more likely to make conservation recommendations (approximately 59%) than studies on species which are not

threatened. Most studies (54% for all island populations and 64% for island restricted species) made no conservation recommendations. There were more studies on non-threatened species (69%) which decreased slightly to 57% when focussing on island restricted species only (Figure 2.6). Around 52% of studies investigated country endemic species, however, when I only took into consideration island restricted species the proportion of endemic species rises to 86%. When considering all studies on island populations, studies of Least Concern species comprised the majority of non-endemic species.

Table 2.1: The number of studies per order of birds categorised by family, genera and species in comparison to the total number in each order.

Order†	No of Studies	No of Families studied	No of Families*	No of Genera studied	No of Genera*	No of Species studied	No of Multi Species Papers	No of Species*
Accipitriformes	22	2	3	11	72	12	3	409
Anseriformes	25	1	3	7	58	12	6	402
Apodiformes	1	1	8	1	155	NA	1	858
Apterygiformes	9	1	1	1	1	2	5	7
Bucerotiformes	2	1	4	1	19	NA	2	103
Charadriiformes	41	9	20	21	99	29	4	649
Ciconiiformes	1	1	1	1	7	1	NA	26
Columbiformes	9	1	1	5	50	5	2	464
Coraciiformes	2	1	6	1	36	2	NA	264
Eurypygiformes	2	1	2	1	2	1	NA	2
Falconiformes	6	1	1	1	11	6	NA	110
Galliformes	26	2	5	11	87	14	3	415
Gruiformes	20	2	6	8	52	10	4	282
Mesitornithiformes	1	1	1	1	2	1	NA	3
Otidiformes	1	1	1	1	10	1	NA	34
Passeriformes	194	48	142	85	1348	102	49	9780
Pelecaniformes	6	3	5	2	36	3	NA	193
Phaethontiformes	1	1	1	1	1	NA	1	11
Piciformes	3	2	7	2	60	3	NA	633
Podicipediformes	1	1	1	1	7	1	NA	32
Procellariiformes	72	4	5	14	30	36	17	232
Psittaciformes	36	5	5	14	93	18	9	566
Sphenisciforme	21	1	1	6	7	9	6	22
Strigiformes	13	2	2	5	29	7	4	386
Suliformes	9	3	4	4	9	5	3	94
†There were 11 papers which studies multiple orders.								
*Obtained from Clements Ebird Data https://ebird.org/science/use-ebird-data/the-ebird-taxonomy								

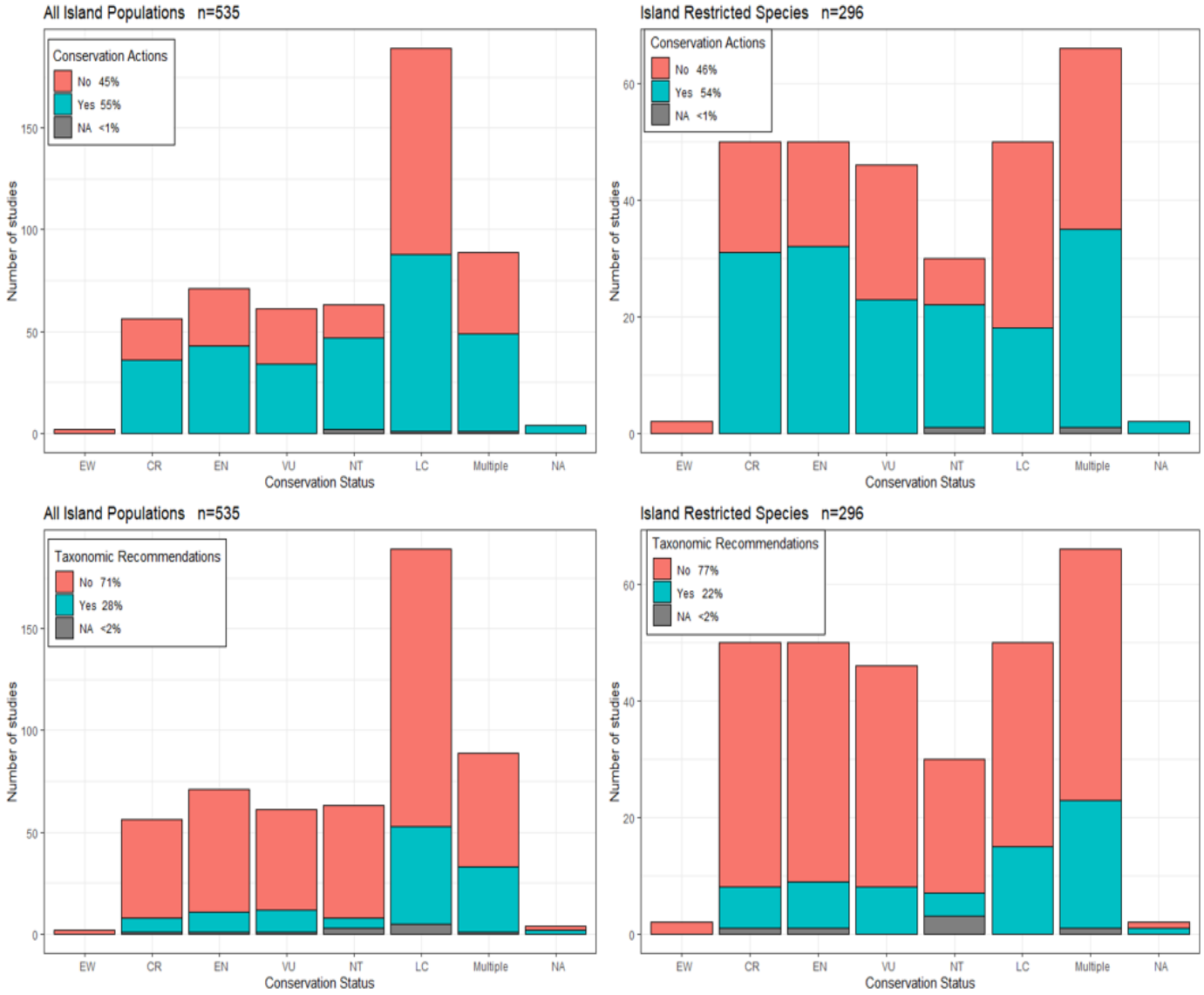

Figure2.5: Comparison between the numbers of published studies for each Conservation Status and the numbers which made conservation and/or taxonomic recommendations for all island populations and for island restricted species. Extinct in the Wild (EW), Critically Endangered (EN), Endangered (EN), Vulnerable (VU), Near Threatened (NT), Least Concern (LC), Not Available (NA)

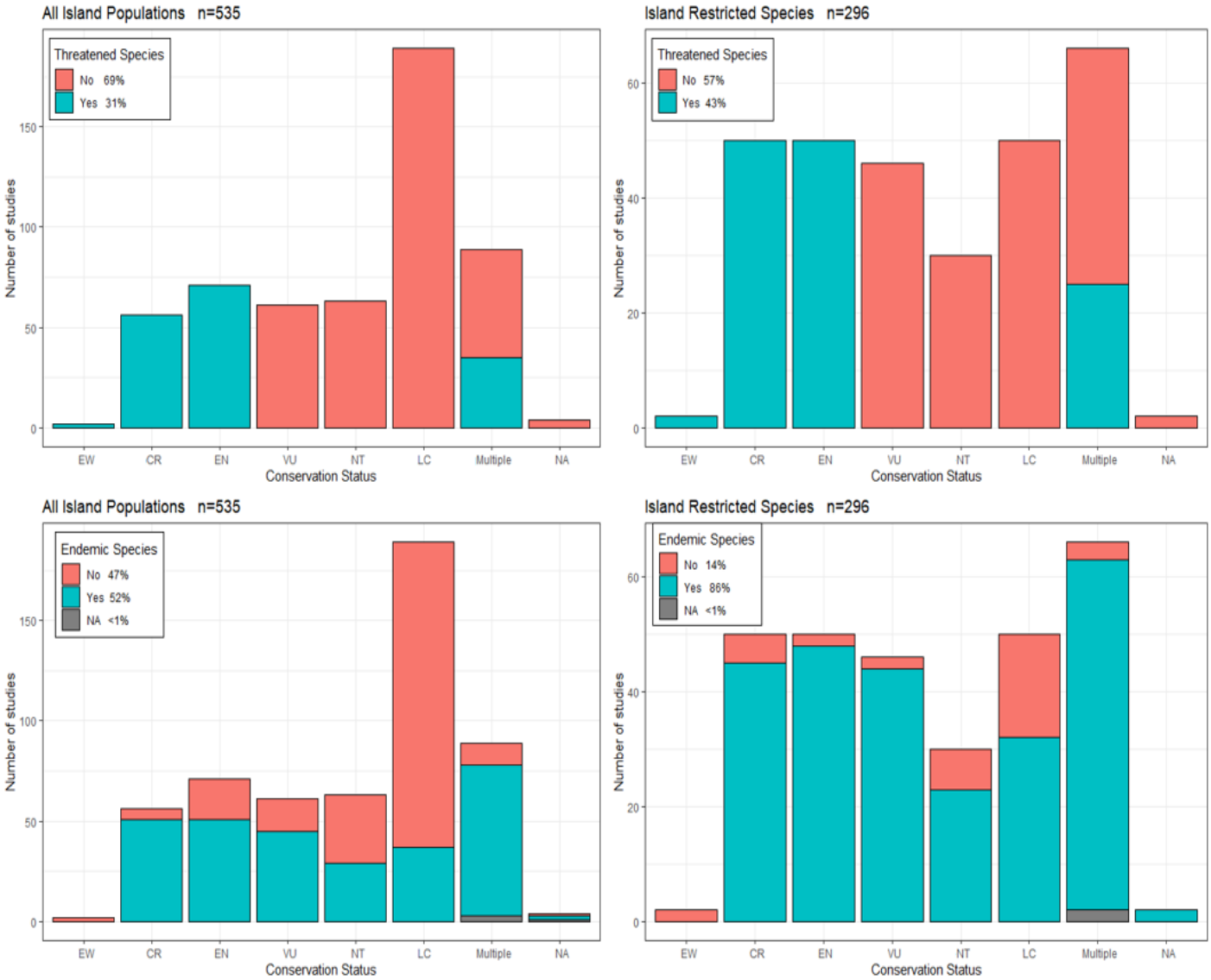

Figure 2.6: Comparison between the numbers of published studies for each Conservation Status and the numbers which were threatened and/or country endemic species for all island populations and for island restricted species. Extinct in the Wild (EW), Critically Endangered (EN), Endangered (EN), Vulnerable (VU), Near Threatened (NT), Least Concern (LC), Not Available (NA)

2.5 Discussion

This study reports an increase in genetic research over time for island populations of birds irrespective of the marker type or sampling method used. This study revealed that the Oceanic realm was the most studied geographical area with the islands of New Zealand being the focus of most of these studies. While there was no particular trend in taxa, the most studied order of birds was Passeriformes which was to be expected given that over half of all bird species are part of this group (Selvatti *et al.* 2015). Over half of studies made conservation recommendations which is higher than other reported values investigating conservation genetic studies (Britt *et al.* 2018).

2.5.1 Methodological trends

Increases in the number of genetic studies for conservation applications are likely to be due to the increasing accessibility of modern molecular genetics making the techniques easier and more affordable (Groombridge *et al.* 2018). Genetic research is become increasingly more important for conservation and genetic assessment and monitoring is now often incorporated into legislation and species recovery programmes which may further explain the increase (Pierson *et al.* 2016; Taylor *et al.* 2017). Despite both criticism around the use of certain markers (Moritz, 1994; Rubinoff & Holland, 2005) and development in new high-throughput technologies (Shafer *et al.* 2015; Britt *et al.* 2018; Mable 2019) an increasing trend was observed in all marker types and sampling methods. While I looked at the use of different marker types it is important to note that a large proportion (almost 40%) used multiple marker types per study.

Both mitochondrial and nuclear markers, as categorised by this study, were used most frequently with no particular difference between the two. The use of mtDNA has been widely criticised for the limited inferences that can be made from the data generated and a preference for the use of newer nuclear markers such as microsatellites and next-generation sequencing methods are often reported (Mable, 2019; Moritz, 1994; Rubinoff & Holland, 2005; Zink & Barrowclough, 2008). As such, a reduction in the use of mtDNA markers over time was hypothesized however, this was not observed

in the data. This may be because it is not always practical or cost effective to look at the entire genomes of multiple organisms (Monsen-Collar & Dolcemascolo, 2010). Mitochondrial DNA can be combined with other genetic data sets to increase its utility, hence the number of papers with multiple marker types, and either in combination or alone is still providing novel genetic information for little known avian species to inform conservation management (Barrowclough & Zink, 2009; Rubinoff & Holland, 2005). Recently mtDNA has been applied to conservation of island birds to reveal cryptic speciation (Saitoh *et al.* 2020), assess genetic diversity (Antaky *et al.* 2020; Arauco-Shapiro *et al.* 2020; Schlesselmann *et al.* 2020), determine population structure (Jackson *et al.* 2020; Forcina *et al.* 2020; Illera *et al.* 2020) and analyse phylogenetic relationships (Lombal *et al.* 2020; Zhu *et al.* 2020). Like mtDNA, nuclear markers can also be used for phylogenies (Pereira *et al.* 2007; Johnson and Weckstein 2011) and assessment of genetic diversity and structure (Omote *et al.* 2012). Nuclear markers have wider applications and have been used to inform conservation management on hybridisation (Cubrinovska *et al.* 2016; Colston *et al.* 2019), kinship and relatedness (Woolaver *et al.* 2013; Bergner *et al.* 2014; Milián-García *et al.* 2015; Galla *et al.* 2019), heterozygosity-fitness (Hagen *et al.* 2011; Monceau *et al.* 2013b) and inbreeding levels (Hagen *et al.* 2011; White *et al.* 2014; Lawson *et al.* 2017) of island birds. Shifts toward genomic techniques may change the direction of conservation genetics from investigations of neutral genetic markers (e.g. microsatellites) to assess additive genetic variation (Clegg & Phillimore, 2010), adaptive potential (de Villemereuil *et al.* 2019b) and functional diversity (Hansson & Richardson, 2005) and it is thought that whole genome analysis will become routine in conservation biology (Postma *et al.* 2009; Groombridge *et al.* 2018; Britt *et al.* 2018; Mable 2019). Given the wide variety of nDNA markers and its broad applications, it may be wise to investigate which nuclear markers are most frequently used, which due to the simplified marker categorisation method used in this study could not be inferred here.

Advances in molecular sexing have allowed simple, reliable and rapid sex determination for birds and is often necessary for management and conservation of endangered wild birds (Bantock *et al.* 2008; Miño and Del Lama 2009; Morinha *et al.* 2012). While the number of studies using molecular

sexing techniques appears to be increasing, a large proportion (approximately 75%) used this technique as an accompaniment to studies using other molecular markers. Molecular sexing is particularly useful for species which are sexually monomorphic (e.g. the Galápagos Petrel *Pterodroma phaeopygia* (Patiño *et al.* 2013) and Japanese Murrelet *Synthliboramphus wumizusume* (Hatakeyama *et al.* 2020)) and for sex determination from unknown and non-invasively collected samples (Huynen *et al.* 2003; Abbott *et al.* 2006; Presti *et al.* 2013).

It is not unreasonable to expect that sequencing of specific functional genes would be popular for island populations of birds given the increased threat of introduced disease to island species (Wikelski *et al.* 2004; Copsey *et al.* 2018; Ralls *et al.* 2020). However, functional genes were the focus of the least studies (3%) in this investigation, although this trend has been increasing in recent years. This may be due to the challenge of deciphering function in relation to the sequence, as well as the long-term investment required to observe adaptive responses over time (Alberts *et al.* 2002; Copsey *et al.* 2018). Such studies of functional genes such as the major histocompatibility complex (MHC) (Bollmer *et al.* 2011; Sutton *et al.* 2013; Stervander *et al.* 2020) and toll-like receptor (TLR) genes (Grueber *et al.* 2012, 2013, 2017) and their implications on immunity have been applied to well-studied species in New Zealand (Grueber *et al.* 2012, 2013, 2017; Knafler *et al.* 2014; Sutton *et al.* 2015). Given the increased susceptibility of island species to disease an increased effort in immunogenetic research for island bird species would be beneficial for the conservation of such at-risk species.

Sampling method is often impacted by the type of sample (blood, feather, egg, tissue, etc.) and the sample source (wild, captive, museum), which are often project dependant meaning that researchers use whatever means are available to obtain samples for analysis (Vili *et al.* 2013; Baus *et al.* 2019). The majority of studies used invasive sampling techniques alone and were most commonly used for wild and contemporary populations. As would be expected studies which used solely non-invasive samples were mostly comprised of museum and ancient samples which are inherently non-invasive at the time of collection (Rohland *et al.* 2004; Horváth *et al.* 2005). I also examined the data

to look for a relationship between conservation status and sampling method, potentially expecting to see non-invasive methods used more for Endangered and Critically Endangered species, however there appears to be no relationship between conservation status and sampling method. This was unexpected as non-invasive sampling is seen as the preferred approach when taking into account ethical and welfare considerations particularly for highly threatened species (Peters *et al.* 2019; Baus *et al.* 2019). Endangered species, as well as being rare, are often elusive and more sensitive to environmental disturbance and as such it is more difficult to obtain permissions and permits for the more intrusive sampling methods which may cause undue stress and risk to the individuals being sampled (Horváth *et al.* 2005; Peters *et al.* 2019). In such instances non-invasive samples including feather, faecal and museum samples (Chan *et al.* 2008; Marrero *et al.* 2008; Ramón-Laca *et al.* 2018) have been used for species identification (Fowler *et al.* 2009), phylogenetic analysis (Martínez-Gómez *et al.* 2015), molecular sexing (Huynen *et al.* 2003) and population genetics (Addison & Diamond, 2011). Given the advances in non-invasive techniques and their usability, a shift towards this type of sampling for endangered species and small populations should be favoured due to ethical considerations particularly as individual identification from such samples becomes more accurate (Taberlet and Luikart 1999; Russello *et al.* 2015).

2.4.2 Geographical distribution

Geographical bias in conservation is well established (Lawler *et al.* 2006; Meijaard *et al.* 2015; Roberts *et al.* 2016; Di Marco *et al.* 2017; Belle *et al.* 2019) and is often attributed to a variety of reasons such as over-representation of wealthy countries (Martin *et al.* 2012) particularly ones with higher Gross Domestic Products (GDP) (Meijaard *et al.* 2015) which have greater access to funds and resources (Waldron *et al.* 2013). Geographical bias may represent the location of scientists (Di Marco *et al.* 2017) or places where genetic monitoring has been built into legal frameworks such as habitat and birds directives for protection of biodiversity (Pérez-Espona, 2017).

A geographical bias in avian island genetic research was also identified by this study showing that the Oceanic realm was by far the most studied geographical area and the most studied group of islands

was New Zealand. Arguably, New Zealand is the leading country in the field of conservation genetic research and its application to the management of populations (Wallis, 2019). This may be due to incentives to make conservation genetics more accessible to practitioners as well as the inclusion of genetic measures in species recovery plans in New Zealand (Taylor *et al.* 2017), as such the disproportionate number of genetic studies from this group of islands may be the consequence of this pragmatic approach to conservation. This has resulted in advances in a wide variety of applications of genetics to the conservation of island restricted birds such as measuring genetic diversity (Boessenkool *et al.* 2007), evaluating hybridisation (Steeves *et al.* 2010), assessment of population structure and gene flow (Baillie *et al.* 2014) and identification of ESUs (Chan *et al.* 2006) along with assessing levels of inbreeding (Hagen *et al.* 2011) and determining variation in immune genes (Grueber *et al.* 2012). Such investigations have also been used for evaluating the success of conservation strategies such as translocations (Ruarus *et al.* 2011), captive-breeding and re-introduction programmes (Bowker-Wright *et al.* 2012). New Zealand provides a unique perspective on conservation genetic research and management which may be due to the high number of "predator-free" sites (Andrews *et al.* 2013), captive-breeding programmes (Ramón-Laca *et al.* 2018), nature reserves and protected sites (Murphy *et al.* 2006) and long-term genetic monitoring (de Villemereuil *et al.* 2019a). Although providing informative case-studies and many success stories, studies from one place, particularly one with such exceptional circumstances may not be applicable or reproduceable for the large majority of counties containing some of the most threatened bird species (Taylor *et al.* 2017).

Other island groups which were frequently studied were the Hawaiian Islands, Californian Channel Islands and Aleutian Islands which largely comprise of US territories. North America has been identified as an area that is heavily studied in conservation research (Lawler *et al.* 2006; Taylor *et al.* 2017) so it is unsurprising that US island territories are well investigated. This may be due to the fact that genetic inclusion is well established in the USA with 82% of species recovery plans incorporating genetic considerations (Pierson *et al.* 2016; Taylor *et al.* 2017). Additionally, US organisation such as the National Oceanic and Atmospheric Administration (NOAA) and the Forest Service regularly

collaborate with geneticists and it is thought that the US more readily incorporates genetics into conservation policy and legislation in comparison to other countries (Taylor *et al.* 2017). While certain countries are leading in this field, providing invaluable case-studies and best practise for other conservationists (Pierson *et al.* 2016; Wallis 2019), the resulting data from only a handful of places may lead to inferences and perceptions that are not representative of more global conditions and thus cannot be applied on a wider scale (Taylor *et al.* 2017).

The Indomalayan realm was one of the least studied areas despite the islands of Southeast Asia being widely documented as an area of conservation priority particularly for bird species due to the high number of threatened species and the prolific illegal songbird trade (Ng *et al.* 2017; Cros *et al.* 2020). Of the studies conducted in this realm a large proportion of these were focused on the Philippines, which although a biodiversity hotspot, is only one area in the vast region of Southeast Asia experiencing high levels of population losses and declines (Ceballos *et al.* 2017). The Caribbean is a globally outstanding conservation priority ecoregion (Latta, 2012) which appears understudied within the Neotropical realm which is comprised largely of studies conducted on species of the Galápagos islands. When considering island restricted species only, approximately half of the conservation genetic research in the Neotropical realm was conducted on the Galápagos. Under-representation of hotspot areas has also been observed by other studies of genetic research in conservation (Belle *et al.* 2019). One reason for the lack of studies in regions such as the Caribbean, despite the high level of biodiversity and endemism, is the literature search used included English-only studies yet in this region a number of languages are spoken including Spanish, French and Dutch which would not have been captured in this search (Lawler *et al.* 2006). Nevertheless, many geographical areas, including those which are of conservation priority, appear to be understudied, and despite potential language limitations in the literature search, studies of native birds in the Caribbean generally remain limited (Nelson *et al.* 2018; Devenish-Nelson *et al.* 2019; Nacional *et al.* 2021). Therefore, regions of high conservation priority, such as the Caribbean and Southeast Asian islands, should be the focus of future conservation genetic research.

2.5.3 Taxonomic patterns

The most studied order of birds was Passeriformes. This was to be expected as this order of songbirds comprises of over half of all bird species (Selvatti *et al.* 2015). The most studied family was Procellariidae which, however, does not fall within the order of songbirds and forms part of the Seabird order (Procellariiformes) which is comprised of just four families (Penhallurick & Wink, 2004). This may be due to the large proportion of seabirds which are island breeding. This was reflected in this study with approximately 70% of seabird studies being island breeding. However, the number of island restricted seabirds represented in this study was less than 27%. The most studied genus was the gadfly petrels, *Pterodroma*; a group of birds which largely comprises of Endangered species (65.7%), declining species (52.9%) and a number of threatened species endemic to New Zealand (17.4%) (BirdLife International, 2022b). When considering only island restricted species, the most studied genus was *Petroica* which is a group made up of Australasian Robins. While no species was studied particularly more than any other the majority which were the focus of single species studies were endemic to New Zealand. With the exception of the widespread Song Sparrow *Melospiza melodia* (LC) - on which there were six papers between 2001 and 2015 investigating island populations particularly on the Gulf islands, British Columbia, Canada and the Channel Islands, California, USA (Keller *et al.* 2001; O'Connor *et al.* 2006; Pruett and Winker 2008; Wilson *et al.* 2009, 2015; MacDougall-Shackleton *et al.* 2011) – the remaining most studied species were endemic to New Zealand. These were the South Island Robin *Petroica australis* a Least Concern species and Kākāpō *Strigops habroptila* a Critically Endangered species with six papers on each. Of the papers on the South Island Robin, three were about immunity (Grueber *et al.* 2012, 2013, 2017) while the others focused on translocation (Heber, 2012) and genetic diversity (Boessenkool *et al.* 2007; Townsend *et al.* 2012). Of the papers on Kākāpō three were focussed on inbreeding and relatedness (Miller *et al.* 2003; Bergner *et al.* 2014; White *et al.* 2014), two were about genetic diversity (Bergner *et al.* 2016; Dussex *et al.* 2018) and one was about immunity (Knafler *et al.* 2014). Other species with relatively high numbers of studies were the Stitchbird *Notiomystis cincta* a Vulnerable species endemic to New

Zealand (Castro *et al.* 2004; Brekke *et al.* 2010, 2011; de Villemereuil *et al.* 2019a, b) and Seychelles Warbler *Acrocephalus sechellensis* a Near Threatened species endemic to the Seychelles (Komdeur *et al.* 1998; Komdeur 2003; Richardson *et al.* 2004; Spurgin *et al.* 2014). Relatively few species are the focus of multiple or long-term studies often meaning only one aspect of their genetics is investigated. Multiple studies on the same species provide information on wider aspects of their genetics, therefore having more conservation implications as well as being useful for long-term genetic monitoring projects (Wan *et al.* 2004; Hu *et al.* 2011).

Despite high levels of species diversity and island specialism, some groups appear to be poorly represented in the literature. Columbidae (Pigeons and doves) are successful island colonizers (Gonzalez *et al.* 2009; Braun *et al.* 2017) and over 20% of Columbidae species are globally threatened and there is an overall decreasing global population trend (BirdLife International., 2021). Of these 78% of threatened columbids are island species (Walker, 2007). Despite this only 10% of genera and a little more than 1% of species of island populations of this order were studied, with only nine papers returned by the search. Of these nine papers, eight were island restricted species. The remaining paper, while strictly not an island restricted species, was on the Barbados population of the Least Concern Zenaida Dove *Zenaida aurita*. Three of these papers were on the Japanese Woodpigeon *Columba janthina*, a Near Threatened species which is endemic to the Ogasawara Islands with all three having a focus on genetic structure (Seki *et al.* 2007; Ando *et al.* 2011, 2014). Perhaps the most well-known endemic island pigeon is the Pink Pigeon which was the focus of a paper investigating the effects of inbreeding and genetic variation which highlighted the importance of long-term genetic management to reduce the impacts of inbreeding and increase a species ability to adapt to future changes (Swinnerton *et al.* 2004). The remaining papers investigated molecular identification from faecal samples (Marrero *et al.* 2008), genetic diversity and gene flow (Young & Allard, 1997), genetic structure (Santiago-Alarcon *et al.* 2006) and molecular phylogeny (Bruxaux *et al.* 2018) for a variety of species only one of which was threatened. Given that Columbidae is a highly threatened family, particularly in Asia, Australia and Oceania, the tropics are recommended as an area for future

conservation genetic research which is an area also highlighted by other studies on pigeon and dove research (Walker, 2007).

A family group with a high level of species diversity with a high proportion of endemics species is Trochilidae (Hummingbirds) (Dalsgaard *et al.* 2018). Around 10% of hummingbirds are globally threatened with 85% of these being endemic species (BirdLife International., 2021). It is the second most species rich order, after Passeriformes, yet less than 1% of genera and species were studied, with only one paper returned in the search. This study investigated the evolutionary history of hummingbirds in Chile; one island endemic and one mainland species and found that competition between the mainland and island restricted species is inhibiting the recovery of the island species and conservation implementations such as habitat restoration and predator control were recommended (Roy *et al.* 1998). Another island specialist group, Mesitornithidae (Mesites), is a family endemic to Madagascar whose phylogenetic relationship to other birds is poorly understood (Gamero *et al.* 2014). This family group is comprised of just three species, all of which have a vulnerable conservation status with declining population trends (BirdLife International., 2021). Again only one paper was returned in the search which characterized 10 specific microsatellite loci for White-breasted Mesites *Mesitornis variegata* as a tool to test the genetic variability and population isolation along with investigating the species social system (Gamero *et al.* 2000).

This study has shown that research on island populations of birds is not proportional to the species diversity in each avian order, although this takes into account all species in an order rather than an island focus. It is well documented that there is a taxonomic bias in conservation with a skew towards more charismatic species, potentially to ensure public interest and funding, regardless of the risks to the most vulnerable species (Donaldson *et al.* 2016; Davies *et al.* 2018; dos Santos *et al.* 2020). Therefore, a more even distribution of research on little-known species would help to reduce potential taxonomic bias arising from an understanding of avian island genetics coming from a handful of species whilst contributing to the ultimate goal of conservationists to preserve all biodiversity (Clark & May, 2002).

2.5.4 Conservation recommendation trends

When evaluating the quantity of studies which make conservation recommendations 55% made conservation and 22% made taxonomic recommendations. This is higher than the reported value of 38% made by Britt *et al.* (2018) which investigated the number of conservation genetic studies which recommended conservation or management actions. In a study which focused on the application of landscape genetics to conservation no papers on birds had conservation applications (Bowman *et al.* 2016). This may be due to the under-representation of birds in landscape genetic studies (Kozakiewicz *et al.* 2018). The focus of this chapter, however, was on conservation genetics of island populations and as there are a disproportionate amount of threatened and endemic species on islands (Tershy *et al.* 2015; Pruett *et al.* 2017; Spatz *et al.* 2017) it stands to reason that more of these studies would make conservation recommendations. The higher reported value by this study may be attributed to the categorisation of conservation recommendations into two groups used by Britt *et al.* (2018): generic (general conservation recommendations i.e. increasing genetic diversity to ensure long-term survival of a population) and specific (direct conservation actions i.e. detailed translocation management strategies). Whereas conservation recommendations for this study encompassed generic measures and were defined as follows: "Studies which make active conservation management recommendations for the focal study populations/ species" as described in Supplementary Material 1: Table 4. The majority of studies were conducted on Least Concern species. This was also noted by Britt *et al.* (2018) who found the majority of conservation genetic studies focussed on species which were of low conservation concern or data deficient. In contrast, when investigating only species restricted to islands a higher proportion of species studied were threatened. This again could be explained by the higher proportion of endangered species which are endemic to islands (Tershy *et al.* 2015).

It is difficult to quantify the application of genetics studies to conservation management. While papers may make specific conservation recommendations it is often unknown whether these actions were carried out. This is primarily due to the difficulty of accessing such information which are often

not available in academic databases as it typically appears in grey literature rather than peer-reviewed journals (Bowman *et al.* 2016). Rather than categorising whether studies did or did not make conservation recommendations, as was my approach and the method used by Britt *et al.* (2018) discussed above, Bowman *et al.* (2016) estimated the proportion of papers that were cited in a search of government websites and resources. One reason for both the difficulty to assess the application of genetic research as well as a reason for why many papers do not make direct conservation recommendations could be this lack of access to central and consistent online resources. Conservation Genetic Resources for Effective Species Survival (ConGRESS) acknowledges this and called for an online resource and community of professionals which would provide not only access to genetic databases, ongoing studies, outcomes of conservation genetic investigation (including from grey literature) but also scientific advice so genetic research can be used in policy and conservation management decisions (Hoban *et al.* 2013a).

Despite the widely acknowledged importance of genetic information being formally recognised by organisation such as the IUCN (Taylor *et al.* 2017) there remains a gap between genetic research and active conservation management (Hoban *et al.* 2013a; Haig *et al.* 2016; Britt *et al.* 2018). While I show that over half of the papers do make conservation recommendations, the remaining studies which do not make direct recommendations do still provide a valuable contribution to increasing knowledge from an academicperspective. Such studies show the importance of genetics in conservation biology by shedding light on taxonomic positions (Melo *et al.* 2017), evaluating the effects of sex-linkage on heterozygosity levels (Brooke *et al.* 2010) and assessing functional immune gene diversity (Stervander *et al.* 2020), regardless of whether they make active management recommendations (Hoban *et al.* 2013a; Britt *et al.* 2018). Irrespective of the contribution, it is important that the field of conservation genetics does not become a solely exercise (Britt *et al.* 2018). Reasons why conservation genetics appears to have less real world applications - such as shortage of access to expertise and funding (Hoban *et al.* 2013a), lack of non-academic co-authors (Britt *et al.* 2018) and uncertainty surrounding datasets obtained from newer techniques (Shafer *et al.* 2015) -

compared to other conservation disciplines needs to be addressed (Bowman *et al.* 2016). A lack of understanding or poor communication of the relevance of genetic research to policy and management (Bowman *et al.* 2016; Haig *et al.* 2016) may reinforce the perception that genetic diversity is not relevant to conservation given that species such as the Little Spotted Kiwi *Apteryx owenii* (Ramstad *et al.* 2013), Pink Pigeon (Swinnerton *et al.* 2004) and Takahē (Grueber & Jamieson, 2011) were all able to recover from extreme genetic bottlenecks and as such genetic factors are seen as a low priority compared to other more imminent threats to biodiversity (Taylor *et al.* 2017). It would be beneficial to prioritise genetic research which directly informs conservation (Hoban *et al.* 2013b) such as measuring gene flow (Wallace *et al.* 2015) and assessing hybridization (Steeves *et al.* 2010) to demonstrate its importance to conservation practitioners.

Focus and funding for conservation genetic studies would be more useful if directed towards both threatened species and projects involving local practitioners actively involved in the management of populations. This could be achieved by incorporating key concepts of genetic management such as effective population size (Morris-Pocock *et al.* 2012), inbreeding (Hagen *et al.* 2011) and population connectivity (Skroblin *et al.* 2012) into conservation policy and government directives. Increasing education and awareness of the role of genetics in the control of disease (Spielman *et al.* 2004) and invasive species (van de Crommenacker *et al.* 2015) among other conservation benefits may highlight the link between genetics and conservation (Taylor *et al.* 2017). Additionally, studies without non-academic co-authors and empirical studies need to better communicate the implications of their research to conservation policies and management strategies in order to make it more applicable to conservation practitioners thus bridging the gap between conservation knowledge and conservation action.

2.6 Conclusion

Genetic research is important for conservation of island bird populations due to their increased risk of genetic isolation (Jouventin *et al.* 2006), inbreeding depression (Swinnerton *et al.* 2004) and reduced evolutionary potential (Lovette *et al.* 1998). The findings of this study reveal

geographical bias, under-representation of island specialist taxa and highlights the gap between genetic research and active conservation management. Therefore, future investigations should focus on under-represented areas, particularly hotspots of biodiversity and endemism, such as the Caribbean and Southeast Asia whilst prioritising threatened species to carry out genetic research which directly informs conservation.

CHAPTER 3 METHOD OPTIMISATION

Photo by Author

Chapter 3: A novel method to optimise the utility of underused moulted plumulaceous feather samples for genetic analysis in bird conservation.

This chapter was adapted and published in *Conservation Genetics Resources* (2019) Volume 12, Issue 3, Pages 457-467 A novel method to optimise the utility of underused moulted plumulaceous feather samples for genetic analysis in bird conservation | SpringerLink

3.1 Abstract

Non-invasive sampling methods are increasingly being used in conservation research as they reduce or eliminate the stress and disturbance resulting from invasive sampling of blood or tissue. Here I present a protocol optimised for obtaining usable genetic material from moulted plumulaceous feather samples. The combination of simple alterations to a 'user-developed' method, comprised of increased incubation time and modification of temperature and volume of DNA elution buffer, are outlined to increase DNA yield and significantly increased DNA concentration ($W = 81$, $p < 0.01$, Cohens's $d = 0.89$). I also demonstrate that the use of a primerless PCR technique increases DNA quality and amplification success when used prior to PCR reactions targeting avian mtDNA. A small amplicon strategy proved effective for mtDNA amplification using PCR, targeting three overlapping 314-359bp regions of the cytochrome oxidase I (COI) barcoding region which, when combined, aligned with target-species reference sequences. I provide evidence that samples collected non-invasively in the field and kept in non-optimal conditions for DNA extraction can be used effectively to sequence a 650bp region of mtDNA for genetic analysis.

3.2 Introduction

Working with cryptic, rare or elusive species can make obtaining invasive samples such as tissue or blood logistically difficult (Mills *et al.* 2000; Horváth *et al.* 2005). Moreover, for endangered and sensitive species, it can be difficult to obtain permits for more intrusive sampling methods, which in some cases are considered unethical (Segelbacher, 2002). In these cases, biological samples such as feathers, hair, buccal cells, faecal matter and shed skin can be collected in the field with minimal disturbance to the study species (Mills *et al.* 2000; Bohmann *et al.* 2014). However, some types of non-invasive sample collection such as buccal swabbing and hair plucking still require trapping and handling of the animal (Broquet *et al.* 2006; Dai *et al.* 2015), and these methods have the potential to cause stress and affect the behaviour of an individual, even if such effects are short term (Broquet *et al.* 2006; Rudnick *et al.* 2009; Dai *et al.* 2015). Highly non-invasive sample collection of material such as moulted feathers, shed skin, faecal samples or eDNA, which can be collected opportunistically in the field, can eliminate the need to interact with the study species (Bayard De Volo *et al.* 2008; Bohmann *et al.* 2014). This is advantageous, particularly for research on sensitive species and ecosystems, as it minimises the level of disturbance to wildlife and prioritises the welfare of individuals being investigated (Dai *et al.* 2015).

Despite the advantages of using highly non-invasive sampling methods, it is often difficult to identify the biological material collected to species-level without genetic analysis (Mills *et al.* 2000; Rudnick *et al.* 2009). This is a particular problem for samples such as feathers, hair and faecal matter that can often come from a variety of species sharing the same habitat with similar somatic features (Waits and Paetkau 2005; Coghlan *et al.* 2012; Ahlers *et al.* 2017). Sample type also has an impact upon the likelihood of successful DNA extraction, for example, large primary, secondary and tail feathers are preferable for obtaining usable genetic material compared to smaller plumulaceous feathers (Dove 2000; Vili *et al.* 2013). Larger feathers are embedded deeper in the body of the bird and so are more likely to retain DNA containing biological material such as epithelial cells (Gebhardt & Waits, 2008; Seki, 2006). Primary feathers in particular can contain the umbilicus blood clot in the shaft of the quill which is a by-product of feather development and, if present, provides a plentiful

source of DNA (Segelbacher 2002; Vili *et al.* 2013). However, opportunistic sample collection methods often remove the ability to select for sample type (Broquet *et al.* 2006). Furthermore, there is often no indication of how long the sample has been in the field and thus exposed to a variety of environmental conditions before collection (Hogan *et al.* 2008; Vili *et al.* 2013). Hot and humid environments provide non-optimal conditions for biological samples intended for genetic investigation (McNally *et al.* 1989; Hanson and Ballantyne 2005) as this can lead to a higher prevalence of decomposing microorganisms such as fungi, mould spores and keratin-degrading microorganisms, which can damage the DNA (Sangali and Brandelli 2000; Vili *et al.* 2013). The increased likelihood of degraded DNA in such samples reduces and often prevents the selection of non-invasively collected samples for use in genetic analyses (Vili *et al.* 2013). Therefore, an improved method is needed to increase the biological value of low quality samples which have been kept in non-optimal conditions; particularly for endangered or elusive species for which invasive sampling methods are not possible (Broquet *et al.* 2006; Hogan *et al.* 2008; Presti *et al.* 2013).

In this study I focused on the utility of non-invasively collected feather samples of the Critically Endangered Grenada Dove *Leptotila wellsi*. I provide a three-step process to allow successful extraction and amplification of mtDNA from plumulaceous feather samples. Firstly, I describe improvements to a user-developed protocol for DNA extraction that increases DNA yield, followed by primerless PCR to improve quality, along with a small amplicon strategy that enabled effective mtDNA amplification using PCR, targeting three overlapping regions of the cytochrome oxidase I (*COI*) barcoding region.

3.3 Methods and Results

3.3.1 Sample Collection

Low encounter rates and cryptic behaviour make surveying the Grenada Dove particularly difficult, requiring intensive monitoring that may cause disturbance (Bolton *et al.* 2015; Rivera-Milán *et al.* 2015; Rusk 2017). Therefore, non-invasive sampling methods were required to obtain samples for genetic analysis with minimal disruption to this Critically Endangered species. Feather samples used for this study were obtained non-invasively, as moulted feathers, and collected opportunistically from known Grenada Dove habitat: Mt Hartman estate and Perseverance, Grenada (Rusk, 2009, 2017). Habitat consists of both dry and mixed broadleaf evergreen-deciduous second-growth forests (Rusk, 2017). This tropical dry forest habitat has a minimum temperature of 22°C and temperatures that can reach up to 32°C, with a maximum rain fall of 259mm in the rainy season and a minimum of 67mm in the dry season (Meteostat 2018; Nelson *et al.* 2018). Due to the opportunistic nature of the sample collection, the feathers used in this study spent an unknown amount of time in the litter bed of this hot and humid environment before collection. Samples were stored in sample bags at 4°C until transportation by airmail to the UK in June 2018. On arrival in the UK, the samples were cleaned with 70% ethanol and stored at -20°C. The sample set (n= 160) used in this study was comprised of 152 plumulaceous (Figure 3.1), three secondary and three primary feathers, as well as two egg shells recovered from the forest floor.

Figure 3.1: Examples of the plumulaceous feathers used in this investigation.

3.3.2 DNA Extraction

The commercially available QIAGEN DNeasy® Blood and Tissue kit was used to conduct DNA extraction. Extractions were carried out as per the 'User-Developed Protocol: Purification of total DNA from nails, hair, or feathers using the DNeasy® Blood & Tissue Kit' (QIAGEN Inc., Crawley) with the following alterations to incubation time and temperature and volume of DNA elution buffer. Feather samples were cut into 1cm pieces directly into a sterile 1.5ml microcentrifuge tube containing the lysis buffers using sterile scissors to increase surface area (Presti *et al.* 2013). The incubation step was increased to 48 hours in order to achieve complete sample lysis on samples that are particularly difficult to digest (Bush *et al.* 2005; Bayard De Volo *et al.* 2008). To increase DNA yield, AE (elution buffer) was heated at 70°C for 10 minutes before use. Buffer AE contains the organic compound Tris (tris(hydroxymethyl)aminomethane, $(HOCH_2)_3CNH_2$)) and EDTA (Ethylenediaminetetraacetic acid ($C_{10}H_{16}N_2O_8$)) which functions to rehydrate the nucleic acids and release DNA from the silica membrane. This process is improved when the DNA and silica are exposed to higher temperatures (Bruns *et al.* 2007; Zhou and Ling 2011; Haddad *et al.* 2017). An elution volume of 100µl was used in a two-step process, giving a final volume of 200µl which was subsequently stored at -20°C. Although using half the recommended volume of elution buffer decreases the DNA yield, the aim was to increase the final concentration of DNA as it is well documented that a higher concentration of starting DNA in PCR reactions improves the likelihood of successful DNA amplification (Kishore *et al.* 2006; Rohland and Hofreiter 2007; Graziano *et al.* 2013).

In order to assess whether the alterations to the protocol had optimised DNA concentration following extraction, I compared the concentration of DNA from samples extracted using the standard manufacturer's protocol and the protocol outlined above. DNA concentrations for samples extracted using the standard protocol were obtained from a feather set that arrived in the laboratory in 2016 (n=50). I used a number generator to randomly select 20 plumulaceous feather samples from the feather set obtained in 2016 and extracted using the standard protocol and from the feather set obtained in 2018 and extracted using the optimised extraction method. The DNA concentration of

each sample was quantified using the Invitrogen™ Qubit™ 3.0 Fluorometer, which has a DNA detection range of 0.50 - 600ng/ml, using dsDNA High sensitivity settings following the manufacturer's protocol (Table 3.1). A Shapiro Wilk test (Shapiro & Francia, 1972) indicated that the data were not normally distributed (Standard Protocol: p=<0.001, Optimised Protocol: p=<0.001) therefore I used a Wilcox test (Wilcoxon, 1992) to compare the difference in DNA extracted when using the standard protocol and the optimised protocol outlined in this study. All analyses were performed in R version 3.5.3 (R Core Team, 2021). The results of the test showed that samples that underwent the optimised protocol had a significantly higher extracted DNA concentration than the standard protocol (W = 81, p-value<0.01, Cohens's *d* = 0.89). Samples that were lower than the detection range of the Invitrogen™ Qubit™ 3.0 Fluorometer were assigned the value 0.49ng/ml. Table 3.1 shows that 30% of the samples extracted using the standard protocol were of too low concentration to evaluate. In comparison, only 5% of the samples that were extracted using the optimised protocol had a concentration below the range of the Invitrogen™ Qubit™ 3.0 Fluorometer. Therefore, both DNA extraction success rate and DNA concentration were increased by the optimised protocol.

Table 3.1: Concentration of DNA (ng/ml) from samples extracted using the standard QIAGEN user developed DNA extraction protocol and samples extracted using the optimised extraction protocol.

Standard Protocol		**Optimised Protocol**	
2016 Samples	**DNA Conc. (ng/ml)**	**2018 Samples**	**DNA Conc. (ng/ml)**
P2	<0.50*	M31	7.45
P4	26.9	M40	10.8
P5	<0.50	M44	1.13
P7	13.0	M46	5.18
P8	<0.50*	M63	3.60
P12	<0.50*	M68	4.60
M1	6.09	M83	1.76
M2	1.28	M95	217.67
M5	0.56	M100	58.17
M8	0.81	M108	1.33
M18	0.82	M114	50.7
M19	7.92	M115	4.64
M22	1.74	M118	<0.50*
M23	1.35	P37	32.1
M28	15.9	P38	307.33
F3	1.32	P39	4.50
F4	1.23	P45	45.43
F5	0.51	P49	34.13
F6	<0.50*	P69	2.23
F8	<0.50*	F12	1.87
Mean	4.12	Mean	39.76
Standard Error	±1.56	Standard Error	±17.82
Median	1.03	Median	4.91
*DNA concentration was too low to read and therefore out of range of the Invitrogen™ Qubit™ 3.0 Fluorometer (range: 0.50 - 600 ng/ml) using dsDNA High sensitivity settings.			

To test that target DNA, rather than that of subsidiary material, had been extracted and to ascertain the potential presence of PCR inhibitors, which are common when using non-invasively collected samples (von Thaden *et al.* 2017; Waits & Paetkau, 2005), avian Cytochrome b (*Cyt b*), was amplified. I designed primers using Primer3 (Koressaar & Remm, 2007; Untergasser *et al.* 2012) based on a *Cyt b* sequence from the White-tipped Dove, whose whole mitochondrial genome is available on GenBank, accession number: NC_015190.1 (Pacheco *et al.* 2011). The Primer3 output for the designed primers is provided in in Appendix II. This species was chosen as it has been suggested, based on the ecology of the species, that it is closely related to the Grenada Dove (Blockstein and Hardy 1989), as molecular phylogenetic analysis had not yet been carried out for the Grenada Dove. The primers were designed to include a 200-250bp region of the *Cyt b* gene, with a length of around 20 bases long, a GC

content of around 50% and melting temperatures that are no more than 5 degrees apart (Dieffenbach *et al.*1993; Naqib *et al.*2019). The chosen primers targeted a 203 bp region of *Cyt b* gene: CYTB_2 Forward: 5'-CTGCCTACTAACCCAGATCCT-3' and CYTB_2 Reverse: 5'-AGGAGCCGTAGTAGAGTCCT-3'. To prevent contamination of samples, PCR was conducted in a PCR hood where tube racks, pipettes and tubes were exposed to UV light for 20 minutes prior to setting up the reaction (Bayard De Volo *et al.* 2008). PCR was conducted using illustra™ PuReTaq Ready-To-Go™ PCR Beads (GE HEALTHCARE; Chicago) with a final volume of 25µl containing ~ 2.5 units of recombinant PuReTaq DNA polymerase, 200µM of dNTP's in 10mM Tris-HCl, 50 mM KCl and 1.5 mM $MgCl_2$ stabilizers, BSA and reaction buffer, 5µl of DNA template and a negative control using sterile H_2O. PCR was conducted on a TECHNE TC-3000 thermocycler (Bibby Scientific Ltd; Stone) using the following conditions optimised for this primer set: initial denaturation at 95°C for 5 minutes, 45 cycles of 95°C for 30 seconds, 48°C for 30 seconds, 72°C for 60 seconds and a final extension at 72°C for 5 minutes. PCR products were separated by electrophoresis (Westermeier, 2005) on a 2% (Mardis & McCombie, 2017) agarose gel (Thermo Fisher Scientific, Waltham) and visualised using a Bio-Rad Gel Doc™ EZ Imager and Image lab 4.0 software (Bio-Rad Laboratories, 2017). DNA extraction using the optimised method resulted in a visible band on the gel at the 203bp target region of avian *Cyt b* for 154 out of 158 (97.5%) of the feathers being used in this study (Table 3.2) thus confirming target DNA rather than subsidiary material had been amplified.

Table **3.2**: Number of successful and failed amplifications of a 203bp target region of avian Cytochrome b gene, recorded by feather type, following DNA extraction using the optimised technique (prior to including the primerless PCR step). Amplification was considered successful if a visible band was present on a 2% agarose gel for the target region.

Feather type	Number of feathers	Number of successful amplifications	Number of failed amplifications
Secondary	3	2 (66.7%)	1 (33.3%)
Primary	3	3 (100%)	0 (0%)
Plumulaceous	152	149 (98%)	3(2%)
Total	158	154 (97.5%)	4 (2.5%)

3.3.3 Primerless PCR

Primerless PCR, also known as 'DNA shuffling' or 'sexual PCR', exposes the DNA template to Taq DNA polymerase, dNTPs and a heating and cooling cycle which serves to denature the sample into smaller fragments which then anneal to each other (Stemmer 1994; Melnikov and Youngman 1999; Brakmann and Schwienhorst 2004; Suenaga *et al.* 2005; An *et al.* 2011). This is known as self-priming and functions to repair DNA damage such as nicks, fragmentation, abasic sites and blocked 3'-ends in degraded DNA samples that may inhibit amplification (Diegoli *et al.* 2012).

Primerless PCR reactions were conducted using illustra™ PuReTaq Ready-To-Go™ PCR Beads with a final volume of 25µl including 5µl of DNA template. Samples were subjected to a PCR cycle with the cycling parameters: initial denaturation at 95°C for 5 minutes, 10 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds and a final extension at 72°C for 5 minutes. A negative control was also generated at this stage by exposing 25µl of PCR reaction mix and no DNA template to the same PCR cycle to ensure no contamination or false amplification occurred during the primerless PCR process.

In order to investigate the efficacy of the primerless PCR process, low quality and quantity samples, as identified by lack of gel band presence, brightness and/or clarity following optimised DNA extraction and amplification (Jacobs *et al.* 2013; Thiel *et al.* 2014) of the 203bp region of *Cyt b* gene outlined above, were chosen. Samples consisted of: 10 plumulaceous feathers, one secondary feather, and one egg shell. Each sample was used as a substrate for the amplification of the barcoding region of the *COI* gene using primer set AWCF1 and AWCintR2 (C1; 328bp) (Patel *et al.* 2010), as described in the Small Amplicon Strategy section below, with and without a prior primerless PCR stage. A standardised dilution factor was used to ensure the same amount of DNA template was used in each primered PCR reaction. Following amplification, the samples were visualised on the UV transilluminator as a comparison for effective amplification with and without exposure to primerless PCR. This can be seen for feather samples in Figure 3.2 and the egg shell sample in Figure 3.3, which show that more bands were present and were more defined after the addition of the primerless PCR

step. Nine of the twelve samples failed to amplify without the addition of primerless PCR but successfully amplified and presented clear bands when exposed to primerless PCR. For example, sample 69 (Figure 3.2; bottom gel image lanes 5 and 12, with and without exposure to primerless PCR respectively) shows a brighter and more defined band is present after under-going the primerless PCR process. The increased prevalence of bands and the improved clarity, brightness and definition of the present bands after primerless PCR indicates the improved quality of DNA after exposure to this process.

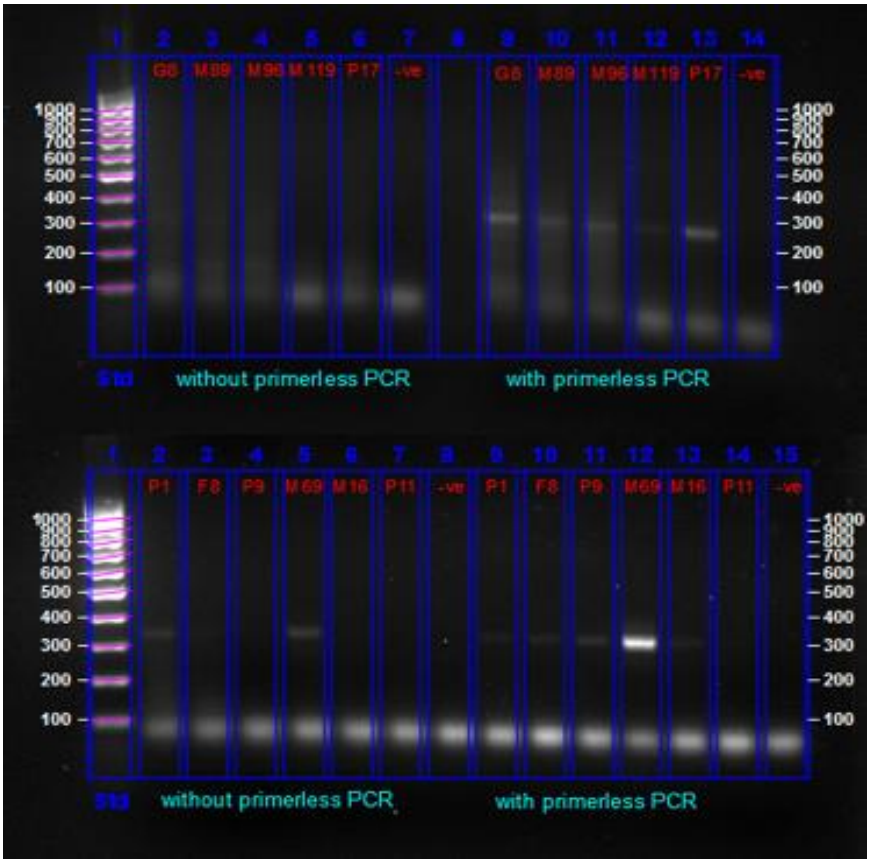

Figure 3.2: Gel image showing the amplification of non-invasively collected plumulaceous feather samples (with the exception one secondary feather (G8)) using primer set AWCF1 and AWCintR2 (328bp) (Patel *et al.*2010) both with and without exposure to primerless PCR.

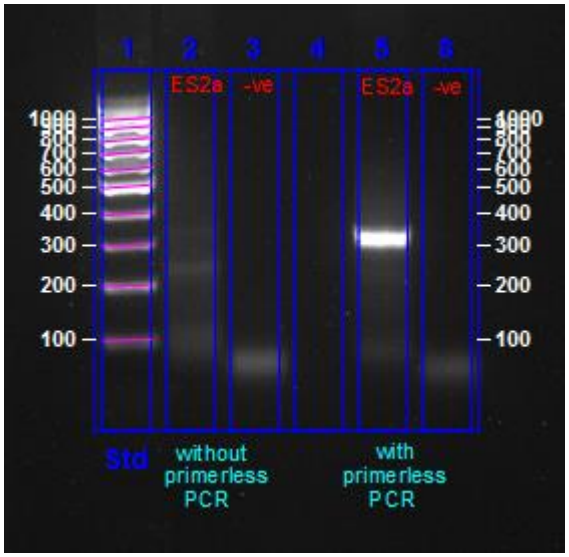

Figure 3.3: Gel image showing the amplification of a non-invasively collected egg shell sample using primer set AWCF1 and AWCintR2 (328bp) (Patel *et al.* 2010) both with and without exposure to primerless PCR.

3.3.4 Small Amplicon Strategy

The third step used was a small amplicon strategy to successfully amplify and sequence three small overlapping amplicons, which were combined to construct a longer and more informative section of the gene. Small amplicons have an increased likelihood of amplification (Broquet *et al.* 2006; Fischer *et al.* 2016; Debode *et al.* 2017) thus I targeted 200-250bp amplicons (Rohland & Hofreiter, 2007; Stiller *et al.* 2009). I performed primered PCR on samples, following inclusion of the primerless PCR step, along with a primerless negative control and a standard negative control, using the following overlapping primer sets, which are known to amplify Columbiforme *COI* barcoding region: AWCF1 and AWCintR2 (C1; 328bp), AWCintF2 and AWCintR4 (C2; 314bp), AWCintF4 and AWCR6 (C3; 350bp) (Patel *et al.* 2010). The PCR reaction was subjected to the same cycling parameters as used in the primerless PCR stage: initial denaturation at 95°C for 5 minutes, 10 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds and a final extension at 72°C for 5 minutes. PCR products were separated and visualised using agarose-gel electrophoresis. All three primer sets successfully amplified DNA from non-invasively collected samples collected for this study (Figure 3.4). Samples were purified using the QIAquick PCR

Purification as per the manufacturer's protocol (QIAGEN Inc., Crawley). Samples were then prepared for sequencing using the Mix2Seq Kit (Eurofins Genomics, Luxembourg) according to the manufacturer's instructions and submitted to Eurofins Genomics, where they were sequenced using Sanger sequencing methods.

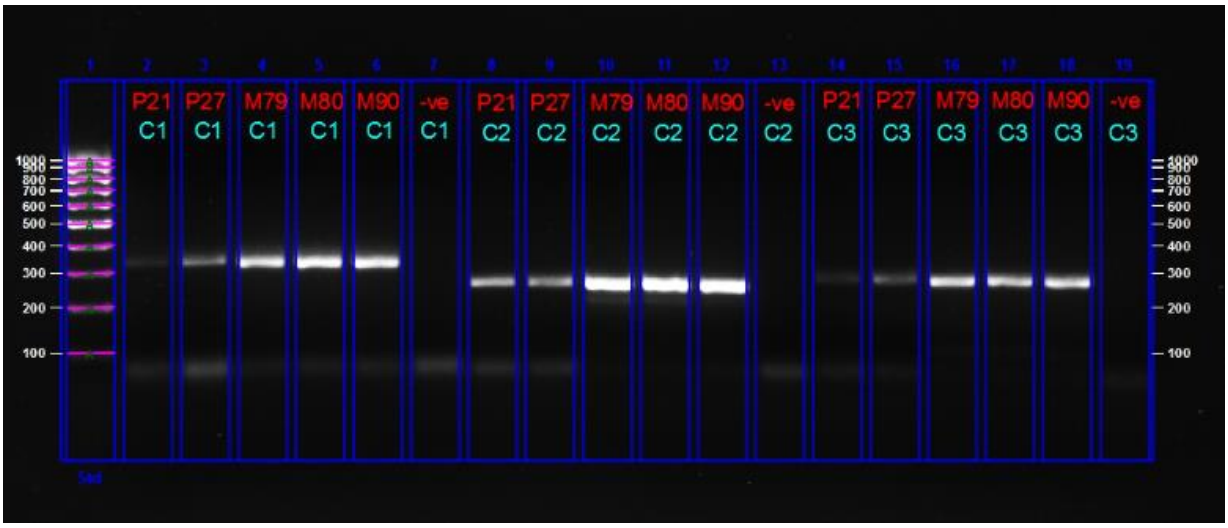

Figure 3.4: Gel image showing successful amplification of the three small amplicons using primer sets AWCF1 and AWCintR2 (C1; 328bp), AWCintF2 and AWCintR4 (C2; 314bp), AWCintF4 and AWCR6 (C3; 350bp) (Patel *et al.*2010) from moulted plumulaceous feathers collected non-invasively and opportunistically in the field.

Sequence data for the three overlapping regions were reconstructed to create an approximately 650bp sequence (Table 3.3). Firstly, consensus sequences were obtained for each amplicon by aligning the forward and reverse sequence data using the contig and consensus mode using Sequencher 5.4.6 (Gene Codes Corporation, 2017) and were used in all further reconstructions and alignments. A consensus calculation using the default Consensus By Plurality setting was used whereby Sequencher determines the consensus line based by majority rule (Gene Codes Corporation, 2011). Obtaining consensus sequence is particularly important when using primerless PCR as random fragmentation and self-priming can introduce artificial recombinants or base errors but this can be counteracted by using consensus sequence (Weber *et al.* 2000). To assemble the longer combined sequence, each of the small amplicons were aligned against a reference sequence and merged to obtain a consensus

sequence. Highly degraded DNA can produce poor read length or low quality base calls in the sequence data, particularly at the ends of the trace producing "messy" end sequences (Bell & Kramvis, 2013). Therefore, most probably due to the highly degraded nature of the samples used in this study, some base inconsistencies were recognised. In these cases, the Sequencher contig (overlapping DNA segments that when combined represent a consensus region of DNA), along with the electropherogram obtained from Eurofins for each sequence (Figure 3.5), were assessed and the base with the consensus by plurality as calculated by Sequencher and the base with the highest quality score as per the Eurofins sequence quality assessment was selected. Due to the non-invasive sample collection method used for these samples, along with the inability to identify the species from the morphological features of the feather alone, each sequence was run in NCBI's Basic Local Alignment Search Tool (BLAST®) to predict species identification (Johnson *et al.* 2008). This search indicated that the sample, presented in Figure 5, was from the Caribbean columbid, White-crowned Pigeon *Patagioenas leucocephala*, with a BLAST total score of 989 and a query coverage of 91% to Genbank sequence JQ175689.1 (Schindel *et al.* 2011). Combined sequence data obtained from the three small amplicons were aligned with the corresponding *COI* barcoding region sequence data from a sample thought to belong to *L. wellsi* (Genbank reference sequence not available for this species) and *P. leucocephala* (accession number JQ175689.1) to confirm the expected nitrogenous base positions, which is particularly important given the possibility of potential base errors introduced by primerless PCR (Weber *et al.* 2000; Johnson *et al.* 2008; Schindel *et al.* 2011). Target-species was confirmed for the 650bp length of the *COI* barcoding region from non-invasively collected plumulaceous feather samples.

Table 3.3: The 650bp sequence obtained for sample M79 aligned with the corresponding *COI* barcoding region sequence data for *Leptotila* sp. and *Patagioenas leucocephala*, labelled with its accession number JQ175689.1 (Schindel *et al.* 2011).

Number of bases	**1**	**80**
Leptotila sp.	TATACCTAATTTTCGGCGCATGAGCTGGCATAGTAGGTACCGCCCTTAGCCTCCTTATTCGTGCAGAACTAGGACNAACC	
M79	.T.GA....AC.TC.ATCCTTC..ACTCCTCCTA.CCT.TT..AC....GAAGC.G.....G.AC.....TG....	
JQ175689.1	..........T..C.....A.....T..C.....C.....T.....-....	
Number of bases	**81**	**160**
Leptotila sp.	CGGTACCCTCCT--AGGAGACGACCAAATCTACAATGTAATCGTCACAGCCCATGCCTTTGTAATAATCTTCTTCATAGT	
M79	GTA.....C...CT..CT.GTA.T.T.GC.C..GCA.G.GC.TC.GT...A..TC...A.CT.CTCCC..CATC.TGCT.G	
JQ175689.1	A.....T..T.---.....T.....C.....T..T.....T..C.....G..	
Number of bases	**161**	**240**
Leptotila sp.	TATACCAATCATGATTGGGGGCTTTGGAAACTGATTAGTCCCACTCATAATTGGAGCCCCTGACATAGCATTCCCACGAA	
M79	.GCT.CTC...CC.A....C.AC-------TTA.CACAA..GC...C.AAA.G------G..CTTT..A..T.ATTT	
JQ175689.1	...G..C.....A..C..A.....C.....C.....C..C.....G....	
Number of bases	**241**	**320**
Leptotila sp.	TAAACAACATAAGCTTCTGACTACTGCCCCCGTCCTTTCTCCTCCTCCTGGCCTCTTCCACAGTTGAAGCTGGTGCTGGA	
M79	.CGG.G-...G....GGCATA.GT..G.A...CA.----------A....TC----------ATCC.C..A.A.	
JQ175689.1	....T.....T.....A..T..A.....C..A.....A.....C.....C.....A..	
Number of bases	**321**	**400**
Leptotila sp.	ACAGGATGAACTGTGTACCCCCCTTTAGCCGGCAACCTAGCCCATGCCGGAGCCTCCGTAGACCTGGCCATCTTTTCACT	
M79	TT....C-.G.CAG....T--T.C...GA.ATG..A.AT.T.CAA..T.ATTGTTAC..C..AT..TT..G.AATGA.	
JQ175689.1	.....C..A.....C...T.....T.....C..A.....C..T..	
Number of bases	**401**	**480**
Leptotila sp.	CCACCTAGCCGGTGTCTCCTCCATCCTAGGGGCTATCAACTTTATTACAACCGCCATTAACATAAAACCTCCAGCCCTGT	
M79	.TT.T.CATG.T.A.GC..AT...AA.C..A.------G....G--GA...T---C..GT----------C.	
JQ175689.1	...T..T..T.....C.....C..G..T.....C.....A.....C.	
Number of bases	**481**	**560**
Leptotila sp.	CACAATACCAAACCCCCCTATTCGTATGGTCAGTTCTCATCACCGCTGTCCTCCTTCTCCTATCCCTCCCAGTCCTTGCC	
M79	..T...-.GGCG.....GACA.A.C..TCC..C.CA.A.A...A...CA.T.	
JQ175689.1	.....G.....G..A.....C.....C..T.....T.....	
Number of bases	**561**	**640**
Leptotila sp.	GCTGGCATCACCATACTTCTTACAGACCGCAACCTGAACACTACATTCTTTGATCCTGCCGGCGGAGGTGACCCAGTACT	
M79	.....A.....A..C.....A.....C.....C..C.....C..C.....T..T.....T.	
JQ175689.1	.....A.....A..C.....A.....C.....C..C.....T..T.....T.	
Number of bases	**641** **653**	
Leptotila sp.	ATATCAGCACCTC	
M79	.....A..T...	
JQ175689.1	.....A..T...	

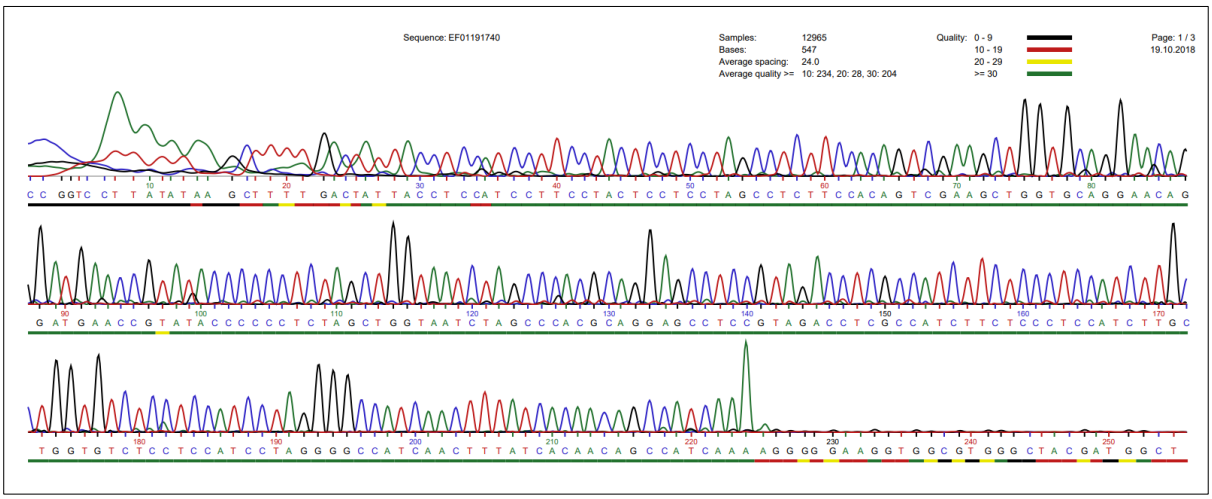

Figure 3.5: Electropherogram example with base quality score as assigned by Eurofins for the forward sequence of *COI*_2

3.4 Discussion

The methods outlined in this study: an optimised user-developed DNA extraction protocol, use of a primerless PCR technique, and a small amplicon PCR strategy, facilitated the attainment of target-species mtDNA sequence data of 650bp in length from non-invasively collected plumulaceous feather samples. Problems arising due to poor quality of DNA extracted from non-invasively collected samples can often dissuade researchers from proceeding with genetic analysis, causing the potential of such samples to go unrecognised (Horváth *et al.* 2005; Speller *et al.* 2011). This can also lead to a preference in invasive as opposed to non-invasive sample collection methods due to the higher confidence in invasive sample quality (Johnson and Clayton 2000a; Harvey *et al.* 2006). The methods I have outlined allow such samples, which previously may have been discounted for genetic analysis, to be successfully used to obtain informative sequence data.

It is widely documented that feather type influences the success and yield of DNA extraction from feather samples (Bayard De Volo *et al.* 2008; Hogan *et al.* 2008). Primary feathers are widely used as a resource for genetic monitoring of wild bird populations, as they often contain a blood clot located in the superior umbilicus of the feather shaft (Horváth *et al.* 2005). Along with assessing the genetic variation within populations (Nichols *et al.* 2001), feather samples have been used to investigate phylogeny and biogeography (Johnson & Clayton, 2000b), and to understand the impact of anthropological processes on genetic structure and gene flow (Fourcade *et al.* 2016) of bird species. However, a study on molecular sexing and microsatellite genotyping of Hyacinth Macaw *Anodorhynchus hyacinthinus* found that despite using larger moulted feathers (with a size greater than 20cm) than in this study, feathers that spent more than seven days in the field and were of poor physical quality, had a low success rate in yielding sufficient DNA for genetic analysis (Presti *et al.* 2013). Nevertheless, I have demonstrated that plumulaceous feathers that are found in the field do not necessarily need to be excluded from sample sets as they can still be utilised to yield informative data.

The use of primerless PCR increased the amplification success in subsequent PCR reactions for samples that had proven difficult to amplify. Bands from samples exposed to primerless PCR were

visually clearer and brighter, which suggests a higher quality of DNA (Hughes-Stamm *et al.* 2011; Jacobs *et al.* 2013; Lucena-Aguilar *et al.* 2016). Primerless PCR has been used in studies of ancient DNA, including that of Weber *et al.* (2000), who increased the successful amplification of ancient DNA from bone samples when investigating the population bottleneck of the Northern Elephant Seal *Mirounga angustirostris*. The same technique was adopted using museum samples from the African Horseshoe Bat *Rhinolophus darlingi* to investigate the phenotypic convergence of its evolutionary history (Jacobs *et al.* 2013). Both studies reported that primerless PCR improved the recovery of DNA from ancient samples but did not comment on differences in DNA quality post primerless PCR (Weber 2004; Jacobs *et al.* 2013). To the best of my knowledge, this study is the first to demonstrate the utility of primerless PCR to increase DNA quality for degraded non-invasive feather samples and demonstrates that this technique can be applied to a variety of non-invasive samples collected in the field.

Although the primerless PCR technique recovers the quality of DNA obtained from the degraded samples this process does not completely repair damaged DNA, particularly when the DNA damage is highly fragmented, which is a common problem with samples kept in non-optimal conditions (Diegoli *et al.* 2012). The varying quality of sequence data at ends of the trace producing "messy" end sequences is a particular problem for overlapping regions resulting in base inconsistencies (Stiller *et al.* 2009). However, this is not specific to the techniques I describe here and is prevalent in many genetic datasets, though it is not widely documented (Sobel *et al.* 2002; Hackett and Broadfoot 2003; Bonin *et al.* 2004). Sequencing error can lead to misidentification of individuals, misinterpretations and erroneous conclusions to be drawn from genetic analysis of relatedness and population structure (Hogan *et al.* 2008). Sequencing error can occur at any stage when obtaining genetic information but there are a number of actions that can be taken to minimise these errors (Bonin *et al.* 2004). The following have been adopted in this study and are proposed for future studies to limit the risk of sequencing error following primerless PCR: 1) the amplification of small amplicons because genotyping error correlates with amplicon size (Vili *et al.* 2013); 2) the inclusion of negative controls (Waits and Paetkau 2005; Alda *et al.* 2007; Boonseub *et al.* 2012); 3) sequence quality

assurance by using only forward and reverse consensus sequences with further analysis and inspection of electropherogram scoring levels; and 4) cross-reading and aligning sequence data with reference samples (Weber *et al.* 2000; Bonin *et al.* 2004). For downstream analyses, it is also recommended to include a sequencing error rate when using genetic data for population and phylogenetic analyses (Bonin *et al.* 2004).

I have outlined methods that achieve successful extraction and amplification of an informative length of mtDNA from non-invasively collected plumulaceous feather samples. The analysis of mtDNA has many practical applications in conservation including species identification as well as presence/absence detection. The high mutation rate of mtDNA in comparison to nDNA allows the identification of ESUs, giving an insight into the phylogenetics of a species (Cronin 1993; Wan *et al.* 2004; Gupta and Bhardwaj 2013). Data pertaining to phylogenetics have multiple conservation implications including evolutionary divergence and speciation, along with phylogeography and rates of change relating to morphology and behaviour of a species (Huang *et al.* 2009). Determining the point at which speciation occurs or defining a species as genetically distinct – known as the phylogenetic species concept – is particularly useful for resolving taxonomic uncertainties, outlining wildlife legislation, and in identifying conservation priorities (Hazevoet 1996; Wan *et al.* 2004; Pellens and Grandcolas 2016; Chen *et al.* 2018). Analysis of mtDNA can be used to make long-term species conservation action plans from an evolutionary perspective (Nielsen *et al.* 2017) and to inform short-term demographic management of populations through identification of population change and connectivity therefore providing information on the effects of habitat fragmentation (Cronin 1993; Moritz 1994; Nabholz *et al.* 2009). Therefore, improved methods of extraction and amplification of mtDNA from non-invasively collected, low quality feather samples, have the potential to extend the applicability of molecular analyses in studies aimed at the conservation of endangered bird species, for which it is typically difficult to obtain high quality samples.

3.5 Conclusion

In conclusion, the optimised user-developed DNA extraction protocol, along with the use of the primerless PCR technique, and a small amplicon PCR strategy, are sufficient to enable DNA extraction and mtDNA amplification from moulted plumulaceous feathers collected non-invasively and opportunistically in the field. This not only provides evidence in support of using non-invasive sampling methods for genetic analyses, in particular when applied to endangered species, but also highlights the utility of biological material kept in non-optimal conditions, which may previously have been discounted (Rawlence *et al.* 2009; Vili *et al.* 2013). Data collected in this manner is informative for species identification, presence/absence detection, population structure and phylogenetic analyses of rare and elusive species (Bonin *et al.* 2004; Marucco *et al.* 2011; Adam *et al.* 2014), all of which are key questions in conservation research.

The published version of this chapter can be seen in Appendix III.

CHAPTER 4 PHYLOGENETICS

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Chapter 4: Phylogenetic placement and life history trait imputation for Grenada Dove *Leptotila wellsi*

This Chapter was adapted and published in *Bird Conservation International* (2022), Published Online, Pages 1-11 Phylogenetic placement and life history trait imputation for Grenada Dove Leptotila wellsi | Bird Conservation International | Cambridge Core

4.1 Abstract

Phylogenetic analyses can be used to resolve taxonomic uncertainties and reconstruct a species' evolutionary history. This can be combined with ecological data to predict missing life history traits which are important for creation of conservation management strategies. This study investigated the evolutionary and life history of the Critically Endangered Grenada Dove *Leptotila wellsi* by estimating its phylogenetic placement and using this new phylogeny to test the accuracy of phylogenetic comparative methods for estimating both documented and unknown life history traits. I extracted DNA from two Grenada Dove samples and obtained sequences from three mitochondrial markers: Cytochrome oxidase I (*COI*), NADH dehydrogenase 2 (*ND2*) and Cytochrome b (*Cyt b*); and one nuclear marker: β-Fibrinogen intron 7 (*β-FIB*). I present the first genetic data obtained for the Grenada Dove. This data identifies the Grey-Chested Dove *Leptotila cassinii* as the species which shares a most recent common ancestor, with an estimated divergence of approximately 2.53 million years ago with the Grenada Dove. Life history trait values for the Grenada Dove predicted from the analyses using phylogenetic imputation are: clutch size=2 (±0.09) eggs, clutches per year=1.4 (±0.81), incubation time=14.2 (±0.75) days, hatching weight=3.8 (±1.05) grams and single imputation: fledging age (genus median)=15.5 days, longevity (genus median)=8.6 years. This study contributes novel information regarding evolutionary history and life history characteristics to inform long-term conservation actions for a Critically Endangered species.

4.2 Introduction

Over 12% of extant bird species are threatened with extinction (Brooks *et al.* 2008), 58.8% of which are island species (Tershy *et al.* 2015). Natural extinction rates are higher in island populations than in mainland populations. Sensitivity to environmental change, predation by introduced predators and small population sizes are all common characteristics among island avifaunas; therefore, island endemic species are at an increased risk of extinction (Frankham, 1998). Taxonomic patterns in extinction risk suggest that families containing high numbers of endemic species are more susceptible to extinction (Lockwood *et al.* 2000). Resolution of unknown taxonomic and evolutionary relationships can therefore contribute to a greater understanding of extinction vulnerabilities among bird groups (Johnson *et al.* 2007; Lockwood *et al.* 2000).

With modern advances in DNA technologies, phylogenetic methods are increasingly used to reconstruct informative relationships within genus and family groups (Johnson *et al.* 2001), reveal evidence for speciation and evolutionary history (Johnson & Clayton, 2000b), determine species presence (Fonseca *et al.* 2010), assess biodiversity (Bell *et al.* 2014), and identify isolated lineages (Robin *et al.* 2010). Such phylogenetic analyses have for example supported the reclassification of Rockhopper Penguin subspecies *Eudyptes (chrysocome) spp*. into different taxonomic species, with concomitant implications for their conservation status (Banks *et al.* 2006).

Advances in systematic biology have allowed phylogenetic information to be combined with phenotypic data to further infer the evolutionary processes, based on the assumption that phenotypic traits within groups of species will be influenced by shared ancestry (Cressler *et al.* 2015; Bastide *et al.* 2018). Recently, phylogenetic comparative methods for missing data imputation have been employed to predict physiological adaptations (Riek & Bruggeman, 2013), conservation status (González-del-Pliego *et al.* 2019) and functional life history traits for data deficient species (James *et al.* 2020). Predictive models are particularly useful for estimation of trait values when species are rare and elusive, locations are remote, and fiscal constraints limit field surveys (Wood *et al.* 2018; Horswill *et al.* 2021). Life history traits such as generation time, longevity, male and female maturity, clutch

size, incubation and fledging time can be used to evaluate the capability of a species to adapt to climate change and habitat fragmentation, as well as being essential to estimate population trends (Mace *et al.* 2008; Pearson *et al.* 2014; Storchová and Hořák 2018; Cuervo and Møller 2019; Horswill *et al.* 2021).

Pigeons and doves are one of the oldest bird lineages, despite this their evolutionary history is still poorly resolved (Pereira *et al.* 2007; Soares *et al.* 2016). It is thought that Neotropical Columbiformes form the most ancient lineage (Johnson and Clayton 2000b; Shapiro *et al.* 2002) and therefore the Neotropics could be the centre of origin of this avian order (Pereira *et al.* 2007). However, Neotropical birds are largely understudied with fundamental taxonomy, conservation status, ecological and life-history data for this group still outstanding (Brooks *et al.* 2008; Londoño-Murcia *et al.* 2010; Latta 2012; Devenish-Nelson *et al.* 2019). Therefore, gathering information about Neotropical species is essential and urgent for conservation of this diverse group of birds (Latta, 2012).

The Neotropical columbid genus *Leptotila* comprises 11 morphologically similar species, of which only one, the Grenada Dove *L. wellsi,* is Critically Endangered (BirdLife International, 2021b). Important details about the species' biology, such as longevity and incubation period, are currently unreported. The Grenada Dove was once considered a subspecies of the Grey-fronted Dove *L. rufaxilla* until reclassified as a distinct species based on morphological and acoustic evidence (Blockstein & Hardy, 1989). However, its genetic distinctiveness and relationships have never been investigated. Anecdotal evidence indicates that the Grenada Dove is closely related to the Caribbean dove *L. jamaicensis* due to morphological similarities (Anthony Jeremiah, Head of Forestry and National Parks Department, Government of Grenada, personal communication).

The aim of this study was to investigate the evolutionary and life history of the Grenada Dove by a) determining whether it is a genetically distinct taxon, b) estimating its phylogenetic placement, and c) using this new phylogenetic information to estimate currently unknown life history traits for this Critically Endangered species. In this study, I extracted DNA from Grenada Dove samples and obtained sequences from three mitochondrial DNA markers: Cytochrome oxidase I (*COI*), NADH

dehydrogenase 2 (*ND2*) and Cytochrome b (*Cyt b*); and one nuclear marker: β-Fibrinogen intron 7 (β-FIB). I used these markers firstly to assess whether there was sufficient divergence in DNA sequence data to consider the Grenada Dove as a genetically distinct taxon, secondly to infer evolutionary history of this species amongst other neotropical Columbidae species, and finally to use phylogenetic comparative methods for missing data imputation to provide life history trait values to inform conservation management decisions.

4.3 Methods

4.3.1 DNA Extraction and PCR

Non-invasively collected eggshell and feather samples (n=2) were collected in 2018 and 2017, respectively. The egg shell sample was provided by head of the Forestry and National Parks Department of Grenada Mr Anthony Jeremiah and collected from beneath a Grenada Dove nest at Mt Hartman estate in May 2018 and the feather samples were provided by forest ranger Mr Doland Francis and obtained from a dead individual found opportunistically in Perseverance dry forest in May 2017. Samples were transported by airmail to the UK in June 2018 where they were cleaned with 70% ethanol and stored at −20°C. DNA barcoding was used to confirm species identification for the samples using methods outlined in Patel *et al.* (2010). As this is the first time Grenada Dove DNA has been obtained, there is no reference sequence available for this species. However, the Grenada Dove is the only member of the genus *Leptotila* resident on Grenada, therefore I conducted a BLAST® search with the data collected here and found that sequences were consistent with those previously obtained for *Leptotila*. I used the optimised method for DNA extraction, using the QIAGEN DNeasy® Blood and Tissue kit and the primerless PCR protocol, outlined in Chapter 2 and Peters *et al.* (2019).

The substrate from the primerless PCR process was used to amplify the mitochondrial markers *Cyt b* (882 bp), *COI (*613 bp) and *ND2* (949 bp) and the nuclear marker *β-FIB* (902 bp). Primer sequences and the corresponding annealing temperatures are given in Table 4.1. PCR cycling parameters for all except the *COI* region were: initial denaturation at 95°C for 5 min, 45 cycles of 95°C for 30 s, 50–60°C

(primer specific, see Table 4.1) for 30 s, 72°C for 60 s and a final extension at 72°C for 5 min; and for the *COI* region: 95°C for 5 min, 10 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 60 s, 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 60 s and a final extension at 72°C for 5 min. Where regions could not be amplified using a single primer set, additional primers were designed using Primer3 (Untergasser *et al.* 2012) allowing a small amplicon strategy to be used where regions were amplified in smaller overlapping sections (as per Peters *et al.* 2019).

Prior to sequencing, samples were purified as per the manufacturer's protocol using the QIAquick PCR Purification kit (QIAGEN Inc., Crawley). Preparation and submission of samples were carried out according to the Eurofins Genomics guidelines for the Mix2Seq kit (Eurofins Genomics, Luxembourg) where sequencing was performed using Sanger sequencing methods. As the primerless PCR technique has the potential to increase the chance of sequencing error, amplification and sequencing were performed in triplicate in order to obtain consensus data for each region (Weber *et al.* 2000; Peters *et al.* 2019). Sequence reconstruction and alignments were conducted using Sequencher 5.4.6 (Gene Codes Corporation, 2017).

4.3.2 Phylogenetics

Sequences from 24 Neotropical columbids with Common Crane *Grus grus* as an outgroup were obtained for all markers from Genbank (Benson *et al.* 2013; Table 4.2). Phylogenetic tree construction was performed using MEGAX 10.2.4 (Kumar *et al.* 2018). I used Maximum Likelihood to fit 24 different nucleotide substitution models and used Akaike information criterion (*AIC*) and Bayesian information criterion (*BIC*) to select the best models (Akaike, 1987; Nei & Kumar, 2000; Schwarz, 1978).

Table 4.1: List of primers and primer sequences used in this study along with the specific annealing temperature and product sizes.

Gene	Primer	Sequence (5'->3')	T_a (°C)	Amplicon size	Reference
COI	AWCF1[†]	CGC YTW AAC AYT CYG CCA TCT TAC C	50*	328bps	Patel *et al.* (2010)
COI	AWCR2	ATG TTG TTT ATG AGT GGG AAT GCT ATG	50*		
COI	AWCF2	ATC GGA GCC CCA GAC ATA GCA TT	50*	314bps	
COI	AWCR4	TTG ATG GCT GTT GTG ATA AAG TTG AT	50*		
COI	AWCF4	TCC TCA ATC CTG GGA GCA ATC AAC TT	50*	352bps	
COI	AWCR6$_t$	ATT CCT ATG TAG CCG AAT GGT TCT TT	50*		
Cyt b	L14841	AAA AGC TTC CAT CCA ACA TCT CAG CAT GAT GAA A	54	1042bps	Kocher *et al.* (1989)
Cyt b	H4a	AAG TGG TAA GTC TTC AGT CTT TGG TTT ACA AGA CC	54		Harshman (1996)
Cyt b	CYTB_32_F	ACC CAG ATC YTA ACA GGY CT	55	350bps	Designed by this study
Cyt b	CYTB_32_R	GTT TGG CCG ATG TAG GGG AT	55		
Cyt b	CYTB_316_F	GTG CCA CAG TCA TCA CCA AC	55	358bps	
Cyt b	CYTB_316_R	GGG TGT AAA GTT TTC TGG GTC T	55		
Cyt b	CYTB_585_F	AAG ACA TCC TCG GCT TCA CA	55	362bps	
Cyt b	CYTB_585_F	GTA GGT GAG GGA GGC AAG TT	55		
ND2	L5216	GGC CCA TAC CCC GRA AAT	60	550bps	Sorenson (2003)
ND2	H5766	RGA KGA GAA RGC YAG GAT YTT KCG	60		
ND2	L5758	GGC TGA ATR GGM CTN AAY CAR AC	54	555bps	
ND2	H6131	CTCTTATTTAAGGCTTTGAAGGC	54		
ND2	*ND2*_420_F	CTG CCC TGC TAC TCT CAA CT	55	408bps	Designed by this study
ND2	*ND2*_420_R	GGT GAG TTC TTG GAT GAT GAG	55		
FIB_BI7	FIB-BI7L	GGA GAA AAC AGG ACA ATG ACA ATT CAC	50	1000bps	Prychitko and Moore (1997)
FIB_BI7	FIB-BI7U	TCC CCA GTA GTA TCT GCC ATT AGG GTT	50		
FIB_BI7	FIB_BI7_15F	CAG AGA CAA TGA TGG ATG GTA CG	55	464bps	Designed by this study
FIB_BI7	FIB_BI7_15R	CCT CAG TAC TGC CAC CCT C	55		
FIB_BI7	FIB_BI7_248F	CCT GCA AGT TAC CAG CCA AA	55	407bps	
FIB_BI7	FIB_BI7_248R	TGA AAG CAG AGC ACA CAG TT	55		
FIB_BI7	FIB_BI7_479F	ACA CTG TCT TGC TTG AGT AGG	55	500bps	
FIB_BI7	FIB_BI7_479R	CTA GAC CTG CCC CAG TAC TG	55		
*PCR parameters contain an initial 10 cycles with the Ta: 55°C followed by an additional 35 cycles at Ta:50°C as described in the methods section.					

Table 4.2: List of species and their Genbank accession number for all sequences used in phylogenetic analysis.

Species	**Complete mtDNA genome**	***β-FIB***	***COI***	***ND2***	***Cyt b***
Colimbina squammata	N/A	AF182651.1[4]	EF373368.1[16]	KJ645747.1[25]	AF483347.1[21]
Columba livia	KF926376.1[11]	AY082415.1[17]	N/A	N/A	N/A
Columbina inca	N/A	AF182650.1[4]	DQ433527.1[8]	KJ645733.1[25]	AF182683.1[4]
Columbina minuta	N/A	AF182652.1[4]	JQ174506.1[20]	KJ645743.1[25]	KJ639100.1[25]
Columbina passerina	N/A	AF182653.1[4]	JN801583.1[26]	KJ645740.1[25]	KF924046.1[19]
Columbina picui	N/A	AF182654.1[4]	FJ027422.1[9]	KJ645737.1[25]	KJ639094.1[25]
Columbina talpacoti	N/A	KJ668681.1[25]	FJ027429.1[9]	KJ645744.1[25]	KJ639101.1[25]
Grus grus	FJ769849.1[10]	DQ881960.1[2]	N/A	N/A	N/A
Leptotila cassinii	N/A	HQ993561.1[6]	JQ175250.1[20]	FJ175707.1[12]	HQ993505.1[6]
Leptotila jamaicensis	NA	AF279706.1[1]	NA	HQ993543.1[6]	AF279716.1[1]
Leptotila megalura	N/A	AF182664.1[3]	FJ027741.1[9]	HQ993545.1[6]	AF483342.1[21]
Leptotila plumbeiceps	N/A	AF279717.1[1]	JQ175252.1[20]	HQ993544.1[6]	AF279707.1[21]
Leptotila rufaxilla	N/A	HQ993560.1[6]	JQ175255.1[20]	AF251546.1[4]	HQ993504.1[6]
Leptotila verreauxi	HM640214.1[15]	HQ993559.1[6]	N/A	N/A	N/A
Patagioenas fasciata	KX902239.1[22]	AF353465.1[5]	N/A	N/A	N/A
Patagioenas leucocephala	N/A	AF182656.1[4]	JQ175689.1[20]	AY274070.1[23]	AF182689.1[4]
Patagioenas plumbea	N/A	AF182658[4]	JQ175696.1[20]	AF251547[4]	AF182691[4]
Patagioenas speciosa	N/A	AF279721[1]	JQ175700.1[20]	AF353442[5]	AF279711[1]
Streptopelia decaocto	NC_037513.1[18]	AF353449.1[5]	N/A	N/A	N/A
Streptopelia roseogrisea	N/A	AF353450[5]	JN801382.1[24]	AF353419[5]	AF353399[5]
Zenaida asiatica	N/A	AF258324.1[4]	DQ433271.1[7]	AF251543.1[4]	AF251533.1[3]
Zenaida auriculata	HM640211.1[15]	AF182667.1[4]	N/A	N/A	N/A
Zenaida aurita	N/A	AF258323.1[3]	JN639032.1[14]	AF251542.1[4]	AF182704.1[4]
Zenaida galapagoensis	N/A	AF258322.1[4]	JQ420133.1[13]	AF251539.1[4]	AF251531.1[3]
Zenaida macroura	NC_031863.1[22]	AY082416.1[17]	N/A	N/A	N/A

Sources: [1](Clayton *et al.*2003), [2](Ericson *et al.*2006), [3](Johnson & Clayton, 2000a), [4](Johnson & Clayton, 2000b), [5](Johnson *et al.* 2001), [6](Johnson & Weckstein, 2011), [7](Kerr *et al.*2006), [8](Kerr *et al.*2007), [9](Kerr *et al.*2009), [10](Krajewski *et al.*2010), [11](Li *et al.*2015), [12](Miller *et al.*2008), [13](Monceau *et al.*2012), [14](Monceau *et al.*2013), [15](Pacheco *et al.*2011), [16](Pereira *et al.*2007), [17](Prychitko & Moore, 2003), [18](Qu, 2018), [19](Santiago-Alarcon *et al.*2014), [20](Schindel *et al.* 2011), [21](B. Shapiro *et al.* 2002), [22](Soares *et al.* 2016), [23](Sorenson *et al.* 2003), [24](M. . Stoeckle, 2011), [25](Sweet & Johnson, 2015), [26](Tavares *et al.*2011)

The Hasegawa-Kishino-Yano (*HKY*) model using a discrete Gamma distribution (+*G*) was the best-supported model for the combined mitochondrial and nuclear DNA dataset of 3346 bp and thus evolutionary analyses were performed using this model with 500 bootstrap replicates (Hasegawa *et al.* 1985). As mtDNA tends to have a larger proportion of variable bases than nDNA (Allio *et al.* 2017), I also inferred the position of the Grenada Dove within the *Leptotila* genus based on the mtDNA and nDNA separately, to provide a comparison between the two marker types, using the following best-supported models as per *AIC* and *BIC*: General Time Reversible model ([+I], 38.01% sites), for a total of 2444 bp of mtDNA (Tavaré, 1986), and Tamura 3-parameter model, for a total of 902 bp of nDNA (Nei and Kumar 2000). I also inferred the position of the Grenada Dove in relation to *L. jamaicensis*, using a reduced genetic dataset using the Hasegawa-Kishino-Yano model (+G, parameter = 0.2496) for a total of 2720 bp of *ND2, Cyt b* and *ϐ-FIB*.

Time-calibrated phylogenies were reconstructed to infer molecular-based estimates of divergence by applying the RelTime-ML method following the protocol outlined in Mello (2018). Fossil calibrations are widely used for molecular dating, but no fossil records are available for the taxa used in this study (Ksepka *et al.* 2015; Peters and McClennen 2015). This study therefore used confidence intervals of minimum and maximum boundary estimations from the TimeTree database, which provides divergence time estimates from published studies integrated with a geological timescale, to calibrate divergence time (Tamura *et al.* 2012, 2018; Kumar *et al.* 2017; Mello 2018).

4.3.3 Life History Trait Imputation

To predict life history traits for the Grenada Dove this study used multivariate phylogenetic comparative methods for missing data imputation implemented in the *Rphylopars* package version 0.3.0 (Goolsby *et al.* 2017) in *R* version 3.6.2 (R Core Team, 2021). This approach uses a multivariate procedure of phylogenetic covariance across species to predict differences in life history traits (Goolsby *et al.* 2017; James *et al.* 2020). I imputed phylogenetic covariance across 24 Neotropical columbid species. Twelve life history traits - clutch size, clutches per year, incubation time, hatching weight, fledging age, longevity, generation length, male and female maturity, no sex body mass

(unreported sex; Myhrvold *et al.* 2015), and male and female body mass - were obtained for each of these species, where available, from primary and grey literature (Appendix IV, Myhrvold *et al.* 2015; HBW Alive 2020).

Available life history trait values for the Grenada Dove were a clutch size of 2 eggs (Rusk, unpublished data), an imputed generation length of 4.2 years calculated using the IUCN generation length calculator (BirdLife International, 2018), a fledging age of 14 days from focal nest observations and an estimate of two or three nesting attempts per year (Rusk, 2010, unpublished data). I imputed these traits to determine whether multivariate phylogenetic comparative methods generated similar values. I fit evolutionary models and used *AIC* and *BIC* as well as assessing model parameters to select the best fitting model. Phylogenetic signals were calculated using a maximum-likelihood approach to quantify the relationship between life history trait values and phylogenetic signal in order to ascertain model precision per life history trait (Penone *et al.* 2014; James *et al.* 2020). I tested the accuracy of the *RPhylopars* method by predicting known values, which were removed individually from the dataset. This focused on the genus *Columbina* as this genus contained the most known data, which allowed us to sequentially remove a total of 20% of these known values for each trait with minimal decrease to the accuracy of predictions (Penone *et al.* 2014). I compared the predicted values from *RPhylopars* to the true value to assess the accuracy of predictions. I also calculated the median values, used in some life-history imputation without explicit phylogenetic information (e.g. Bird *et al.* 2020), for fledging age and longevity as this was considered a more accurate representation for these traits (discussed below **4.4.2 Life History Trait Imputation**).

4.4 Results

4.4.1 Phylogenetics

There were no polymorphisms observed between the two Grenada Dove sequences. There were 27 nucleotide polymorphisms across 3346 bp that were unique to the Grenada Dove and not present in any other *Leptotila* species used in this investigation, consisting of 21 transition mutations,

two transversion mutations and one transition/transversion mutation (depending on the *Leptotila* species) in the mtDNA and three transitional mutations in the nDNA β-FIB gene. The phylogenetic tree constructed using concatenated sequence data - *Cyt b* (882 bp), *COI* (613 bp), *ND2* (949 bp), and *β-FIB* (902 bp) - for Neotropical columbids indicates that the Grenada Dove falls within the monophyletic group formed by *Leptotila* species (Figure 4.1a.). The Grenada Dove forms an individual branch suggesting a single lineage not shared by any of the other *Leptotila* species, thus indicating that this species is a genetically distinct taxon. The Grenada Dove shared a most recent common ancestor with its sister group containing *L. cassinii* and *L. plumbeiceps* approximately 2.53 million years ago (mya) (Figure 4.2a). The smallest genetic distance (p=0.0303) where 100 nucleotides were polymorphic (Table 4.3) was identified between the Grenada Dove and *L. cassinii*. The phylogenetic tree built with the reduced data and containing *L. jamaicensis* (Figure 3) still shows a sister relationship between the Grenada Dove and *L. cassinii* and *L. plumbeiceps*.

The mitochondrial phylogenetic trees shown in Figures 4.1b and 4.2b suggests that the Grenada Dove shared its most recent common ancestor with *L. cassinii* and *L. plumbeiceps* approximately 2.18mya*,* which is consistent with the phylogenetic tree constructed with the concatenated data set in Figure 4.1a. The nuclear phylogenetic trees shown in Figures 4.1c and 4.2c suggests that the Grenada Dove shared its most recent common ancestor with *L. megalura* and *L. rufaxilla* approximately 2.75 mya*,* dissimilar to the most recent common ancestor shown by the phylogenetic trees constructed with the combined (Figure 4.1a and 4.2a) and mtDNA (Figure 4.1b and 4.2b) datasets. Analysis of the *β-FIB* gene relevels an indel (Appendix V) in *L. cassinii* and *L. plumbeiceps* sequences which is not shared by the Grenada Dove or any of the other *Leptotila* species included in this study.

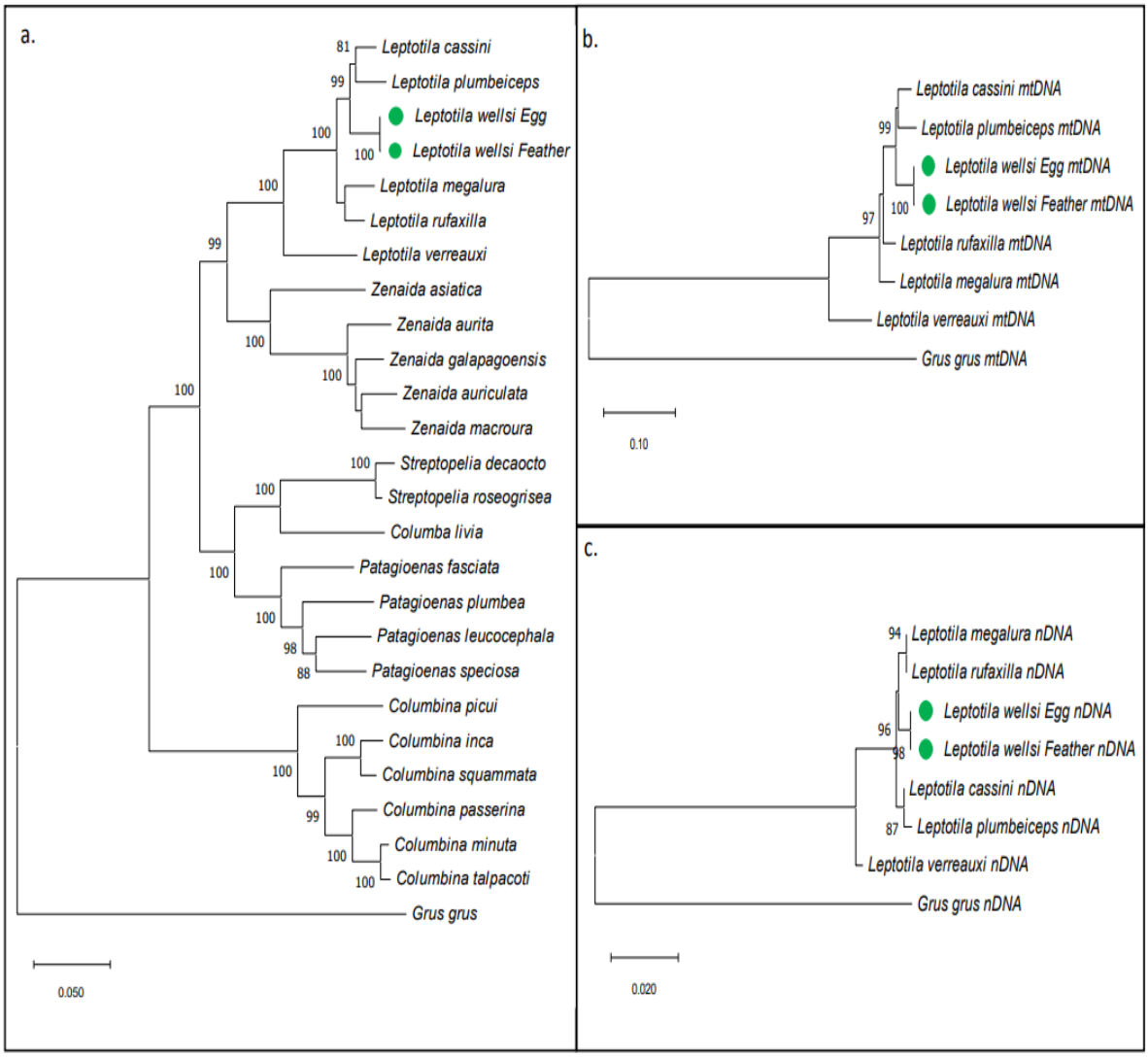

Figure **4.1:** Phylogenetic analysis for 24 Neotropical columbid species and outgroup using the Maximum Likelihood method. Grenada Dove sequences indicated by the circular symbol; a. all mitochondrial and nuclear sequence data estimated using the Hasegawa-Kishino-Yano model (+G, parameter = 0.2281); b. mtDNA using General Time Reversible model ([+I], 38.01% sites); c. β-fibrinogen using the Tamura 3-parameter model. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site and nodes supported by bootstrap values of >80%

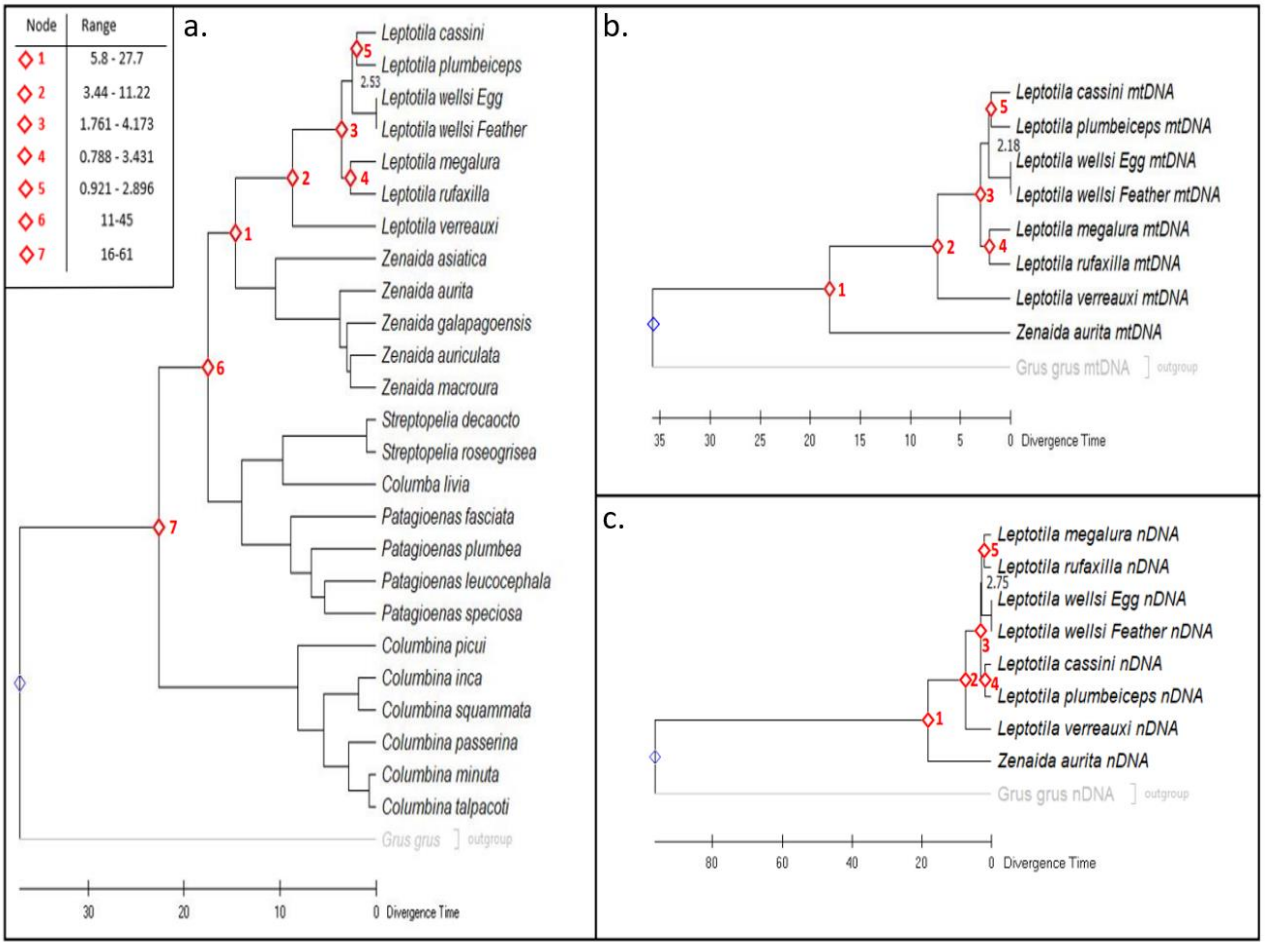

Figure **4.2:** Time-calibrated phylogenies for *Leptotila* species and outgroup using the RelTime-ML method a. all mitochondrial and nuclear sequence data estimated using the Hasegawa-Kishino-Yano model (+G, parameter = 0.1547), b. mtDNA General Time Reversible model ([+I], 35.97% sites) c. β-fibrinogen using the Tamura 3-parameter model. Number scale indicates million years ago (mya). Nodes indicate 7 estimated divergence time calibration points of estimated divergence time ranges in million years ago (mya) obtained using the TimeTree resource (Kumar *et al.* 2017)

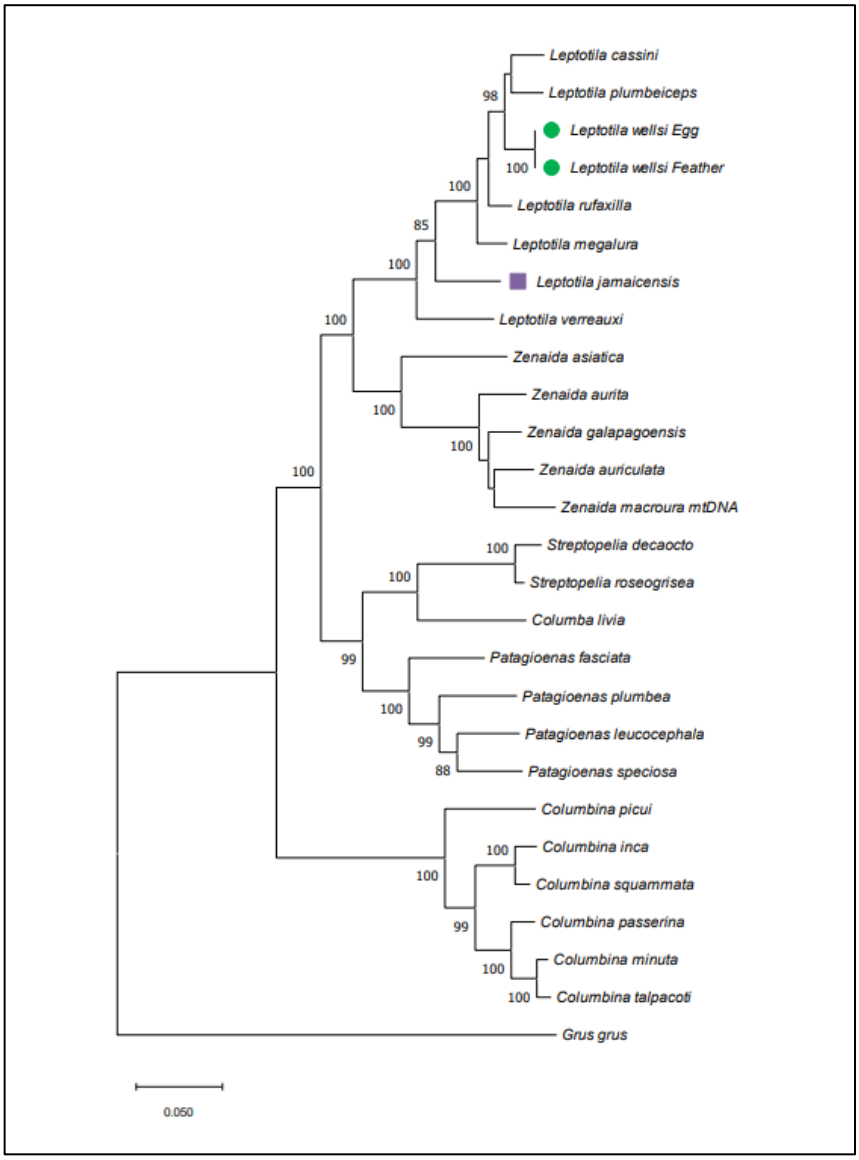

Figure **4.3:** Phylogenetic analysis for 25 Neotropical columbid species and outgroup using the Maximum Likelihood method. Grenada Dove sequences indicated by the circular symbol and *Leptotila jamaicensis* are indicated by a square using the Hasegawa-Kishino-Yano model (+G, parameter = 0.2496). Tree drawn to scale, with branch lengths measured in the number of substitutions per site and nodes supported by bootstrap values of >80%

Table 4.3: The number of substitutions per site (p-distance) and the number of base differences (polymorphic sites) between sequences for a total of 3346 bp positions in the final dataset. *2720 bp positions in the final dataset.

Species	P - distance	Number of polymorphic sites
Leptotila cassinii	0.0303	100
Leptotila megalura	0.0424	140
Leptotila plumbeiceps	0.0339	112
Leptotila verreauxi	0.0719	237
Leptotila rufaxilla	0.0383	126
*Leptotila jamaicensis**	0.0622	167

4.4.2 Life History Trait Imputation

Strong phylogenetic signals were revealed for clutch size, no sex body mass, and male and female body mass, whereas weak phylogenetic signal was detected for clutches per year, incubation time, hatching weight, fledging age, longevity and generation length (Appendix VI). Model validation (Figure 4.4) showed lower variation relative to each trait around seven of the twelve life history trait predictions including clutch size, clutches per year, incubation time, hatching weight, fledging age, longevity and generation length and were therefore considered accurate. However, the remaining five life history trait predictions including male and female maturity, no sex body mass, and male and female body mass showed a high level of variation from the true value and therefore were discounted. Despite suggested trait accuracy from the model validation for fledging age and longevity, I report large standard error values in addition to weak phylogenetic signal, and as such, genus medians were considered a more accurate representation for these traits. Known values for generation length for *Leptotila* are themselves imputed using the IUCN generation length calculator, and thus were discounted this trait from the results. Overall, six life history trait values were predicted for the Grenada Dove with confidence (Figure 4.5) using multivariate and single imputation procedures: a. clutch size=2 (±0.09 *S.E.*) eggs, b. clutches per year=1.4 (±0.81 *S.E.*), c. incubation time=14.2 (±0.75 *S.E.*) days. d. hatching weight=3.8 (±1.05 *S.E.*) grams, fledging age=15.5 days (genus median) days, f. longevity=8.6 years (genus median).

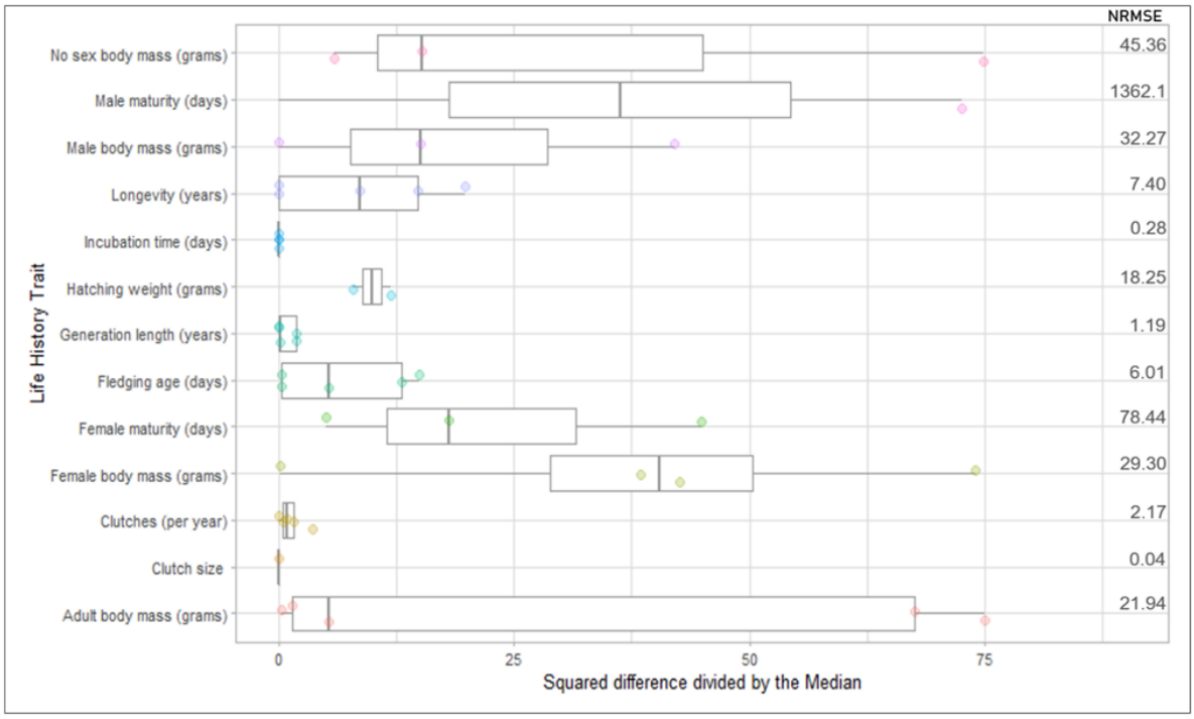

Figure 4.4: Plot displaying the squared difference between the EB model predicted value and the true value divided by the trait median using the genus *Columbina* along with Normalized Root Mean Squared Error [NRMSE (Penone *et al.*2014; James *et al.*2020)]. Raw data are represented by the ° and the first, second and third quartile are represented by the box plot. Mean Squared Error per life history trait

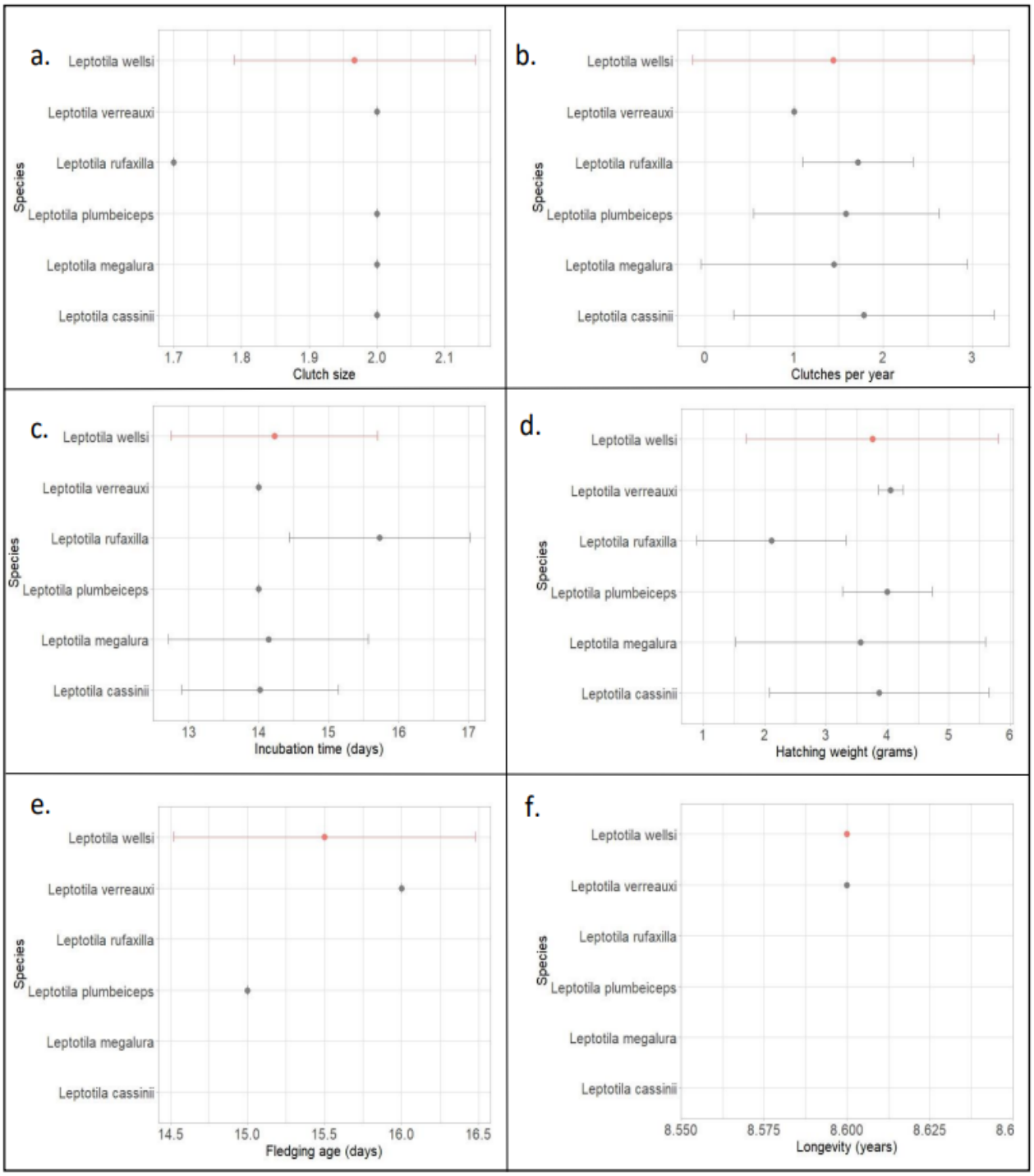

Figure 4.5: Predicted trait values with upper and lower 95% confidence intervals for the Grenada Dove *Leptotila wellsi* in comparison with other Leptotila species for the following lift history traits using the EB model: a. clutch size, b. clutches per year, c. incubation time, d. hatching weight; and median estimates: e. fledging age, f. longevity. Species of interest highlighted red. Known trait values represented by individual grey point

4.5 Discussion

This study reports the first genetic data obtained for the Grenada Dove. The results show that this species formed an individual branch on the phylogenetic tree and is as divergent as other species within the tree. Phylogenetic results support a sister relationship with the Grey-chested Dove *L. cassinii* along with the Grey-headed Dove *L. plumbeiceps*, whose ranges span Central America into Colombia (BirdLife International, 2022b). I provided estimations for six previously unpublished life history traits for the Grenada Dove some of which, such as clutch size and clutches per year, have been found to be correlated with extinction risk (Parlato *et al.* 2015).

4.5.1 Phylogenetics

Results of this study indicate that the Grenada Dove is a genetically distinct taxon as it forms an independent branch within the *Leptotila* monophyletic group, signifying single lineage. Previously, the Grenada Dove was thought to be a subspecies of *L. rufaxilla* (American Ornithologists Union, 1983). However, this has been shown to be based on errors reported when describing its morphology (American Ornithologists Union, 1983; Blockstein & Hardy, 1989; Bond, 1973; Lack & Lack, 1973; Schwartz & Klinikowski, 1963). These findings support the work of Blockstein and Hardy (1989) whose morphological analysis and sonographic data lead to the reclassification of the Grenada Dove as a distinct taxon. As such, the Grenada Dove can now confidently be confirmed as a unique species, which provides additional support for the IUCN conservation status of a Critically Endangered species (BirdLife International, 2022b; Rusk, 2017).

*Leptotila cassinii w*as the species with the smallest genetic distance from the Grenada Dove (p=0.0303) with 100 bp nucleotide polymorphisms based on concatenated sequence data and phylogenetic reconstruction. The most recent common ancestor was shared approximately 2.53 mya between *L. cassinii, L. plumbeiceps,* and the Grenada Dove. However, when the mtDNA and nDNA are considered separately these results show disparity. The mtDNA shows the same relationship displayed by the concatenated data described above, but nDNA analysis revealed that the most recent common

ancestor was shared between *L. rufaxilla, L. megalura,* and the Grenada Dove. The mito-nuclear incongruence may have arisen for a variety of reasons; a larger amount of mtDNA sequence data was used providing a stronger phylogenetic signal, the differing characteristics between these markers such as differential lineage sorting and linkage disequilibrium, all of which can lead to ambiguous patterns of variation and evolutionary inferences (Hurst & Jiggins, 2005; Rubinoff & Holland, 2005). Overall, this study identifies *L. cassinii* and *L. plumbeiceps* as the species sharing the most recent common ancestor with the Grenada Dove, however, caution is recommended here due to the disparity between the mitochondrial or nuclear phylogeny. Nevertheless, this result was unexpected as *L. cassinii* is predominantly a Central American species and has a larger geographical distance from Grenada than *L. rufaxilla* which the Grenada Dove was previously thought to be a subspecies. This study reveals a genetic difference (p=0.04) and 126 nucleotide polymorphisms between the Grenada Dove and *L. rufaxilla* (American Ornithologists Union, 1983). These findings support the work of Blockstein and Hardy (1989) whose morphological analysis (showing differing morphological traits such as cinnamon underwing on primary feathers, a greater extent of white abdomen and a lesser extent of white on the tail tips) and sonographic data (no response was made by the Grenada Dove to *L. rufaxilla* playback presentations) led to the reclassification of the Grenada Dove as a distinct taxon. Anecdotal evidence suggested the Grenada Dove is closely related to *L. jamaicensis,* however these results show that the Grenada Dove shares common ancestors with four other *Leptotila* more recently than its shared ancestor with *L. jamaicensis* and with a greater genetic distance and number of nucleotide polymorphisms in comparison.

The evolutionary history of the *Leptotila* genus was inferred by Johnson and Weckstein in (2011) using a molecular phylogeny to investigate the role of the Central American land-bridge in avian dispersal-driven diversification events. Their results show that this genus diverged into two clades at a time that coincides with the closure of the Isthmus of Panama forming the Central American land-bridge. Their study suggests that the northern clade dispersed southwards from North America stepwise through Central America, crossing the land-bridge into South America, while the southern

clade, containing *L. rufaxilla* and *L. cassinii,* dispersed in the opposite direction from South America across the Panama Isthmus (Johnson & Weckstein, 2011). These results indicate that the Grenada Dove is placed within the southern clade and not with the northern clade containing *L. jamaicensis.* I hypothesise that there was a shared evolutionary history with an upwards dispersal event from South America by the ancestor shared by *L. rufaxilla*, *L. cassinii* and the Grenada Dove with a range expansion to Central America and the southern islands of the Lesser Antilles. Dispersal over the barriers presented by the South American Andes and the Caribbean Sea implies reduced gene flow between the ancestral populations and eventual isolation. This is supported by the absence of the indel in the Grenada Dove nDNA, suggesting this mutation occurred after divergence from the shared common ancestor with *L. cassinii* and *L. plumbeiceps*. These data support the hypothesis that allopatric speciation, due to isolation of the Grenada Dove and likely exposure to differing selective pressures, may have resulted in the evolution of Grenada Dove as a distinct species.

4.5.2 Life History Trait Imputation

These analyses were able to predict six life history values for the Grenada Dove. Values for clutch size, clutches per year and fledging age agreed with documented values from focal observations (Rusk, 2010, unpublished data). With model estimations of only 2 eggs per clutch and 1.4 clutches per year the Grenada Dove has a relatively slow breeding rate, which has been shown to be correlated with extinction risk (Robinson *et al.* 2010; Hutchings *et al.* 2013). Given the IUCN generated a 4.2 year generation length and with a low predicted longevity (genus median = 8.6 years), these values suggest this species will be slow to recover after a major population decline (Bird *et al.* 2020). As not all traits are phylogenetically conserved (Kamilar & Cooper, 2013), I tested for phylogenetic signal and found that signal strength varied amongst traits. Clutch size and generation length both exhibited strong phylogenetic signals, as is expected owing to their correlation with body mass, which indicates phylogenetic conservatism of these life history traits (Kamilar and Cooper 2013; Calhoon *et al.* 2014). All other traits exhibited low phylogenetic signal strength, which is not uncommon in traits such as these that often evolve as a response to differing environmental conditions (Kamilar and Cooper 2013;

Martin *et al.* 2018). Although this model validation has suggested that many of the predicted values are accurate, these results have to be used cautiously, and would benefit from validation from field data (Ando, 2019).

When comparing predicted values for life history traits for the Grenada Dove and *L. cassinii* (the species revealed to share the a most recent common ancestor and the smallest genetic distance) and *L. rufaxilla* (the species it was once considered a sub-species of) this study found that a clutch size of two and more than one clutch per year was shared between all three species. The Grenada Dove and *L. cassinii* both having a hatching weight of over 3 grams and an incubation time of around 14 days whereas *L. rufaxilla* has a slightly lower hatching weight of around 2 grams and a slightly longer incubation time of 15 days. While there is no great difference in life history traits between studied members of the *Leptotila* genus, as expected, it is found that the species with life history traits most similar to the Grenada Dove is *L. cassinii* which is the species also revealed to have the smallest genetic distance. Again, these predicted values have to be used cautiously since life-history traits of island species are, in some cases, known to differ from those observed in mainland species (Beauchamp, 2021; Clegg, 2010).

4.5.3 Conservation implications

The more favourable conservation status of the other *Leptotila* species examined in this study may be attributed to their much larger ranges and availability of suitable habitat (BirdLife International, 2022b). This study reinforces the need for increasing suitable habitat available to the Grenada Dove to reduce extinction risk, both through habitat restoration and protection, as has been outlined in the recovery and action plan for this species, as well as identification of additional habitat for establishing new populations and enabling population increase (Rusk *et al.* 2008).

4.6 Conclusion

The Grenada Dove is a genetically distinct taxon that diverged from its most recent common ancestor approximately 2.53 mya. I identify *L. cassinii* as the species with the highest genetic similarity to the

Grenada Dove. This study used the phylogenetic relationship of the Grenada Dove amongst other Neotropical Columbidae in combination with life history data to predict estimates for fundamental life history traits previously undocumented in the literature, contributing to an improved insight into the biology of the Grenada Dove. Although Columbifomes are one of the oldest and largest bird lineages, there are still unresolved evolutionary histories and taxonomic queries within this order (Soares *et al.* 2016). These results provide insight into the phylogenetic placement of a novel Columbidae species and contribute to a greater understanding of this diverse avian family. This information is likely to be important for conservation efforts on Grenada and will allow conservationists to make more informed management decisions in order to mitigate the extinction risk and protect this Critically Endangered species.

The published version of this chapter can be seen in Appendix VII

CHAPTER 5 POPULATION GENETICS

Photo by Author

Chapter 5: Mitochondrial genetic analysis of the Critically Endangered Grenada Dove *Leptotila wellsi* reveal isolated populations with low mtDNA diversity

5.1 Abstract

Small isolated population fragments with low genetic diversity are at risk of genetic erosion and so have an increased threat from extinction. I assessed the genetic diversity of populations of the Critically Endangered Grenada Dove by a) assessing mtDNA genetic diversity in the two areas of occupancy, b) defining the number of haplotypes present in Grenada Dove at each site and c) evaluating evidence of isolation between sites. I used non-invasively collected samples from two locations: Mt Hartman (n=18) and Perseverance (n=12). DNA extraction, primerless PCR and PCR were used to amplify 1,751 bps of mtDNA from two mitochondrial markers: *ND2* and *Cyt b*. Haplotype diversity (*h*) of 0.4 and nucleotide diversity (π) of 0.4 and two unique haplotypes were identified. Of the two haplotypes identified; the most common haplotype (haplotype A = 73.9%) was observed at both sites and the other (haplotype B = 26.1%) was unique to Perseverance. Selective neutrality tests Fu's *F*s, =0.78 and Tajima's *D* = 0.83 were performed which are positive and not significant. The F_{ST} value = 0.71 suggests that there is marked genetic divergence between the two populations. This study shows low mitochondrial genetic diversity, a non-expanding population and shows clear evidence for genetically isolated populations. These results lead to recommendations for habitat protection and augmentation of gene flow by translocation in order to increase genetic resilience and diversity with the ultimate aim of securing the long-term survival of this Critically Endangered species.

5.2 Introduction

Genetic diversity is important in ensuring the long-term survival of a species whether this is adaptive variation, known to enhance the evolutionary potential of a species to adapt to ecological change, or neutral variation, known to mitigate against the threats of inbreeding and genetic drift increasing population viability (Baillie *et al.* 2014; Kahilainen *et al.* 2014; Frankham *et al.* 2017). Preserving high levels of genetic variation and preventing the fixation of deleterious alleles are crucial for the long-term persistence of populations (vanDyke 2008; Kahilainen *et al.* 2014). Failure to preserve genetic diversity of a species can lead to a variety of negative consequences (Frankham 2005; Kahilainen *et al.* 2014) such as inbreeding depression (Wright *et al.* 2008), higher frequency of harmful alleles (Robinson *et al.* 2018), decreased resistance to disease (Spielman *et al.*2004) and increased parasite load and abundance (Whiteman *et al.* 2006). Low genetic diversity, whether neutral or adaptive, is thought to increase the risk of extinction in wild populations (Kahilainen *et al.* 2014).

Loss of genetic diversity is common in small populations (Frankham *et al.* 2019) as the rate at which genetic variation is lost is determined by the effective population size (Ne) which is the number of individuals that contribute to the next generation (Frankham *et al.* 2017). Island populations are generally small and isolated, with their range restricted by the physical boundaries of the island they inhabit (Frankham *et al.*2017; Groombridge *et al.*2018). As such, low levels of genetic variability are often observed in island restricted populations (Hudson *et al* .2000). Johnson *et al.* (2009), investigated the genetic diversity of *Haliaeetus* species, which found that the island endemic Madagascar Fish-eagle *Haliaeetus vociferoides* had much lower genetic diversity than the continental species studied. Island populations are often formed by a small founder event and are more prone to bottlenecks, genetic drift, inbreeding depression and reduced genetic diversity (Frankham 1997; Grant *et al.* 2001; Dudaniec *et al.* 2011; Gonzalez-Quevedo *et al.* 2015) which, as a result, can reduce the viability of populations (Kahilainen *et al.* 2014). The Red-crowned Crane *Grus japonensis* on Hokkaido island lost genetic diversity after a population bottleneck coupled with long-term isolation and it is thought that it is unlikely to recover its genetic variation (Akiyama *et al.* 2017). Conservation of genetic

diversity is therefore imperative in ensuring the long-term survival of a species, particularly in small and isolated populations, which are at an increased risk of loss of genetic diversity (Lacy 2000; Frankham *et al.* 2019).

One island bird species, with both a small population and a restricted range, is the Critically Endangered Grenada Dove (Rivera-Milán *et al.* 2015; Rusk 2017). It is thought that geographical isolation may have may have played a role in the evolution of the Grenada Dove (Peters et al., 2022; Chapter 4), however, due to the stepping-stone dispersal pattern often observed in Caribbean islands it is expected that some gene flow would have been present early-on following island colonization (Bellemain et al., 2008; Clegg, 2010; Sendell-Price et al., 2021). The historical distribution of this species was once widespread across dry coastal zones in the North-East, West and South-West of Grenada, including off-shore islands (Baptista et al., 2020; Bond, 1973; Clarke, 1905; Collar et al., 1992; Lack & Lack, 1973). The current distribution is restricted to the West and South-West of Grenada with some records of single occurrences outside of these areas in the South-East (Rusk 2009, 2017; Rivera-Milán *et al.* 2015). However, populations of the Grenada Dove are presumed to be limited to sites in the West (Perseverance, Woodford, and Beausejour area) and Southwest (Mt Hartman Estate and surrounding area) of the Island which are approximately 1.6km^2 and 2.23km^2 respectively (Rusk 2009, 2017; Bolton *et al.* 2015).

Along with the populations' small and restricted range (Bolton *et al.* 2015) populations of the Grenada Dove appear to be isolated. The Southwest and West populations, although thought to be historically connected, are now believed to be isolated due to the 9km^2 development - including the islands capital city St Georges - which separates the two (BirdLife International, 2021b; Blockstein, 1991; Rusk, 2017). Genetic variation in isolated populations is often lost over time as a result of long-term genetic drift (Bollmer *et al.* 2005; Reynolds *et al.* 2015). Isolated populations pose a survival risk as they are more vulnerable to inbreeding and genetic erosion (Wright *et al.* 2008; Major *et al.* 2021). In addition to population isolation the Grenada Dove is also threatened by habitat loss from industrial and commercial development such as road construction, housing and agricultural developments

(Bolton *et al.* 2016; Rivera-milán *et al.* 2015; Rusk, 2017). A serious threat to the Grenada Dove population is a proposed resort construction at Mt Hartman estate and the surrounding area, which holds approximately 48% of this species according to the census conducted in 2007 (Rusk, 2008, 2010). Isolation and fragmentation through habitat loss reduce population connectivity and results in lack of gene flow due to dispersal barriers (VanderWerf *et al.* 2010; Cros *et al.* 2020). Both dispersal patterns and population connectivity of this species are unclear (Baptista *et al.* 2020). In populations with low levels of connectivity and gene flow, minor alleles are easily lost to genetic drift, which are fundamental causes of loss of genetic diversity making populations more prone to extinction (Asai *et al.* 2006; Major *et al.* 2021). Given the small population (Rivera-Milán *et al.* 2015; Rusk 2017) and fragmented habitat (Bolton *et al.* 2015) of this Critically Endangered island species, isolated populations and lack of gene flow pose a large risk of inbreeding, genetic erosion and decreased survival. Therefore investigation of genetic variation of this species is important to determine whether genetic intervention is needed when creating species recovery plans and population-based management strategies (Mischler *et al.* 2018; Major *et al.* 2021).

The aim of this study was to assess the haplotype diversity of the Grenada Dove across its range with the purpose of increasing the understanding of this species to inform evidence-based conservation management at a population level. I did this by a) assessing mtDNA genetic diversity of Grenada Dove in the two areas of occupancy, b) defining the number of haplotypes present in Grenada Dove at each site and c) evaluating evidence of isolation between sites. In this study I extracted DNA from non-invasively collected feather samples from Grenada, designed a species-specific marker to identify target species, obtained sequences from two mitochondrial markers (*ND2* and *Cyt b*) and used universal sexing primers to molecularly sex the samples. I used this information to infer whether populations were isolated based on site specific haplotypes and to assess the level of genetic diversity both within and between these populations.

5.3 Methods

5.3.1 Sample collection

Non-invasive sampling methods were required to ensure minimal disturbance to this Critically Endangered species. Moulted feathers and egg shell samples were collected opportunistically in the months of May and July between 2014 and 2018 by members of the Forestry and National Parks Department of the Government of Grenada from two known dove habitats: Mt Hartman Estate and Perseverance, Grenada. Information such as site and date were recorded, and where possible the location using a handheld GPS, to accompany each sample. Feather samples (n=194) and egg shell samples (n=3) were stored at 4°C. until transportation to the UK in September 2016 and June 2018 where they were cleaned with 70% ethanol and stored at − 20°C.

5.3.2 Species identification

Species-specific primers were designed based on a unique region of *COI* sequence obtained from specimens provided by the Head of the Forestry and National Parks Department, Mr Anthony Jeremiah, and Forest Ranger, Mr Doland Francis. The designed primers: LW4_F: GTTATACCAATCATGATTGGG and LW4_R: GTTAATGGCGGTTGTAATAAAG targeted a 261 bp region of *COI* and were tested against seven species, including 3 closely related species found on Grenada, to ensure amplification of Grenada Dove DNA only (Appendix VIII). Optimised PCR cycling parameters were as follows: initial denaturation at 95 °C for 5 min, 45 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 5 min. All non-invasively collected samples were processed using this primer set. Positive Grenada Dove identification was confirmed using species-specific primers. Samples displaying no species-specific band were excluded from further investigation on the grounds that they were either poor quality or not from the target species. Demographic and biological information can be used to assist individual identification (Johnson *et al.* 2007a; Russello *et al.* 2015), so I used temporal and spatial information provided with the samples - site location, GPS information and sample collection dates - and molecular sexing data in an effort to distinguish between different

individuals. Using standard sequence identification methods, sequenced samples were queried in NCBI's BLAST® nucleotide database to confirm species identification (Jarman *et al.* 2004; Ross *et al.* 2008; Ovaskainen *et al.* 2010; Zhuang *et al.* 2012; Price *et al.* 2015).

5.3.3 DNA Extraction and PCR

DNA extraction was carried out with the QIAGEN DNeasy® Blood and Tissue kit (QIAGEN Inc., Crawley) using the optimised protocol outlined in Chapter 2 and Peters *et al.* 2019. PCR was used to amplify mitochondrial markers: *ND2* and *Cyt b* using a small amplicon strategy (Patel *et al.* 2010; Peters *et al.* 2019) with primers shown in Table 5.1. Degraded samples underwent a primerless PCR process prior to amplification (Peters *et al* .2019). PCR was conducted using illustra™ Hot Start Mix RTG PCR Beads (Cytiva; Sheffield) with a final volume of 25 µl containing ~ 1.75 units of recombinant PuReTaq™ DNA polymerase, 1.65 µg of Hot Start Activator, 10 mM Tris–HCl (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM $MgCl_2$, 200 µM of dNTP's, BSA and reaction buffer, 8 µl of DNA template and a negative control. The following PCR cycling parameters were used: initial denaturation at 95°C for 5 min, 45 cycles of 95°C for 30 s, 50-60°C (primer specific, see Table 5.1) for 30 s, 72°C for 60 s and a final extension at 72°C for 5 min. PCR purification was carried out using the QIAquick PCR & Gel Clean-up Kit as per the manufacturer's protocols (QIAGEN Inc., Crawley). Samples were visualised on a 1% agarose gel (Thermo Fisher Scientific, Waltham) using a Bio-Rad Gel Doc™ EZ Imager and quantified using Image lab 4.0 software (Bio-Rad Laboratories 2017). Samples were prepared and submitted to Eurofins Genomics following the Mix2Seq kit (Eurofins Genomics, Luxembourg) instructions for sequencing using Sanger sequencing methods. Sequence assembly was performed using Sequencher 5.4.6 (Gene Codes Corporation, 2017).

Table 5.1: List of primers and primer sequences used in this study along with the specific annealing temperature and product sizes.

Gene	Primer	Sequence (5'->3')	T_a (°C)	Amplicon size	Source
Cyt b	CYTB_32_F	ACC CAG ATC YTA ACA GGY CT	55	350bps	(Chapter 4; Peters *et al.* 2022)
Cyt b	CYTB_32_R	GTT TGG CCG ATG TAG GGG AT	55		
Cyt b	CYTB_316_F	GTG CCA CAG TCA TCA CCA AC	55	358bps	
Cyt b	CYTB_316_R	GGG TGT AAA GTT TTC TGG GTC T	55		
Cyt b	CYTB_585_F	AAG ACA TCC TCG GCT TCA CA	55	362bps	
Cyt b	CYTB_585_F	GTA GGT GAG GGA GGC AAG TT	55		
ND2	L5216	GGC CCA TAC CCC GRA AAT	60	550bps	(Sorenson, 2003)
ND2	H5766	RGA KGA GAA RGC YAG GAT YTT KCG	60		
ND2	L5758	GGC TGA ATR GGM CTN AAY CAR AC	54	555bps	
ND2	H6131	CTCTTATTTAAGGCTTTGAAGGC	54		
ND2	*ND2*_420_F	CTG CCC TGC TAC TCT CAA CT	55	408bps	(Chapter 4; Peters *et al.* 2022)
ND2	*ND2*_420_R	GGT GAG TTC TTG GAT GAT GAG	55		

5.3.5 Molecular Sexing

Samples were sexed using the universal avian sexing primers P8 and P2 (Griffiths *et al.* 1998) and cycling parameters outlined in Çakmak *et al.* (2017). The PCR product was visualised on a 3% gel to determine sex based on banding pattern of one band for males and two bands for females (Griffiths *et al.* 1998; Morinha *et al.* 2012; Çakmak *et al.* 2017). Sexing was performed in triplicate to ensure accuracy (Chang *et al.* 2008).

5.3.6 Analysis of mtDNA Genetic Diversity

Genetic analysis were conducted using the PopGenome package (Pfeifer *et al.* 2014) in R version 3.6.2 (R Core Team, 2021). To assess genetic diversity, I identified biallelic sites and mutation type, determined haplotypes using the haplotype count function, along with calculating haplotype (*h*) and nucleotide (π) diversity. To test for selective neutrality Fu's *F*s (Fu, 1997) and Tajima's *D* (Tajima, 1989) were conducted. Wright's fixation index (F_{st}) was performed to test for inter- and intra-nucleotide

differences among sites. Sequences with low average quality score *ND2* (n=7) and *Cyt b* (n=1) were removed from mtDNA genetic analyses.

5.4 Results

The final sample set used for each site was as follows: Mt Hartman (n=18: feathers (n=16); egg shells (n=2)) and Perseverance (n=12: feathers (n=12); Appendix VIIII, representing approximately 18% of the total Grenada Dove population. I conducted a BLAST® search for each sample and found that sequences obtained for this study were consistent with previously published sequences for *Leptotila* (*ND2*: >94.56% Per. Ident with 882 total score for *Leptotila plumbeiceps* Accession Number: HQ993544.1; *Cyt b* : >95.56% Per. Ident with 970 total score for *Leptotila cassinii* Accession Number: HQ993505.1 (Johnson & Weckstein, 2011)). This indicates the DNA fragments amplified in this study represent the intended target species, which is the only *Leptotila* resident on Grenada. Molecular sexing was carried out (Appendix X) for all of the samples and indicated the sample set was comprised of 5 females and 18 males (Mt Hartman: females=5, males=11; Perseverance: females=0, males=7) with the sex unknown for the remaining 7 samples.

5.4.1 mtDNA Genetic Diversity

I amplified a total of 1,751 bps of mtDNA (n=23) which comprised of 965 bps of *ND2* and 786 bps of *Cyt b*. Two unique haplotypes were identified using the 1,751 bps combined gene regions with a haplotype diversity (*h*) of 0.4 and a nucleotide diversity (π) of 0.4. The most common haplotype (A = 73.9%) was found at both sites, and the second haplotype (B = 26.1%) was found only in Perseverance (Figure 5.1). The results of the selective neutrality tests, performed to determine population expansion, were Fu*'s F*s, = 0.78 and Tajima's *D* = 0.83, which are positive and not significant.

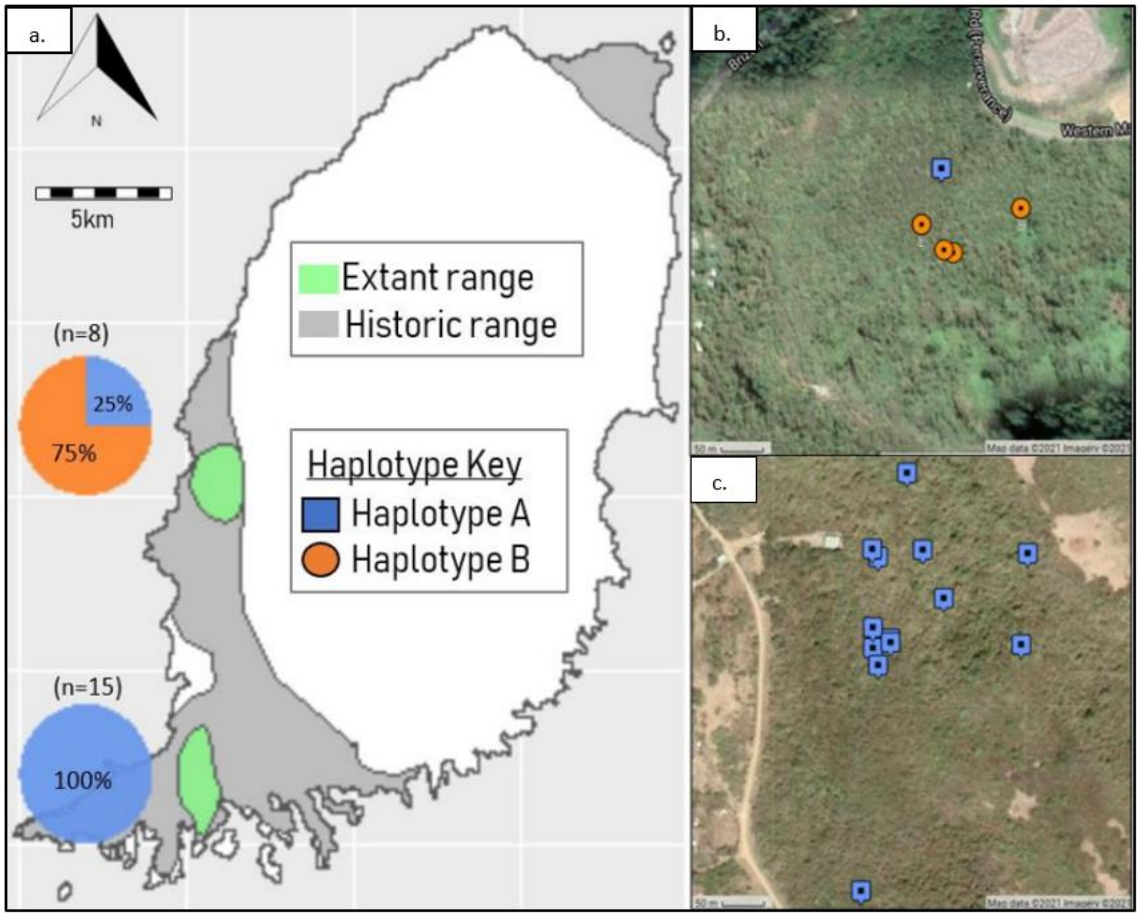

Figure 5.1: a. Grenada Dove haplotype distribution among sampled localities on Grenada b. haplotype distribution within Perseverance and c. haplotype distribution within Mt Hartman

5.4.2 Gene Comparison

Variation within the 965bp of *ND2* (n=23) included one single transitional polymorphism at the 882 bp position and as such two haplotypes were present, no variation was detected within the single haplotype identified within the 786bps of the *Cyt b* gene (n=29).

5.4.3. Population Comparison

Observed within population diversity using the 1,751 bps combined gene regions was higher at Perseverance (h=0.43, π=0.43) in comparison to Mt Hartman which showed no variation in any of the samples from that site (Table 5.2). Mt Hartman (n=15) was comprised solely of haplotype A: 15 (100%) whereas Perseverance (n=8) comprised of haplotype A: 2 (25%) and haplotype B: 6 (75%) (Figure 5.1). F_{ST} between the two locations was 0.71.

Table 5.2: Genetic diversity indices based on 1,751 bps of mtDNA for each population.

Site	No of Individuals	No. of Haplotypes	No. of unique haplotypes	No of polymorphic sites	Haplotype diversity (*h*)	Nucleotide diversity (π)
Perseverance	8	2	1	1	0.43	0.43
Mt Hartman	15	1	0	0	0	0
Overall	23	2	-	1	0.4	0.4

5.5 Discussion

The Grenada Dove population has low haplotype diversity based on the examination of two mitochondrial genes. The two extant sites that constitute the entire range of the species are likely isolated from each other, as shown by the unique haplotype found in the West but not the Southwest population. Molecular sexing revealed a male-sex bias in the samples used in this study. Given estimated population size of around 160 individuals (Rivera-Milán *et al.* 2015), along with isolated populations, this species is at increased risk of inbreeding depression and a further loss of genetic diversity due to genetic drift, which can lead to decreased fitness and survival (Wright *et al.* 2008; Brekke *et al.* 2010; Houston *et al.* 2015; Price *et al.* 2015). Therefore, management interventions at a population level are required to prevent further loss of genetic variation in the future (Kahilainen *et al.* 2014; Price *et al.* 2015).

5.5.1 mtDNA Genetic diversity

The number of haplotypes (2) and nucleotide polymorphisms (1) obtained in this investigation are low in comparison to similar mtDNA studies carried out on other Columbidae species for example: Zenaida Dove 11 haplotypes (Monceau *et al.* 2013a); Japanese Woodpigeon 27 haplotypes (Seki *et al.* 2007); Eurasian Collared Dove *Streptopelia decaocto* 52 haplotypes (Bagi *et al.* 2018). In this study I performed Fu's *F*s and Tajima's *D* tests, analysis for population expansion (Seki *et al.* 2007; Monceau *et al.* 2013a; Bagi *et al.* 2018), which revealed results indicative of a non-expanding population. This is consistent with population counts of territorial singing males from 1987-2015 which show a somewhat

stable population trend (Blockstein 1991; Rivera-Milán *et al.* 2015; Rusk 2017). Whilst neutral genetic data does not give us an insight into phenotypic variation and short-term adaptability (Reed and Frankham 2003; Kahilainen *et al.* 2014; Le Gros *et al.* 2014; Copsey *et al.* 2018), neutrality tests can shed light on population expansion and bottlenecks which can both influence patterns of genetic variability (Ramírez-Soriano *et al.* 2008; Pfeifer *et al.* 2014; Bagi *et al.* 2018). While I have taken every precaution to ensure data was collected from different individuals, due to the non-invasive and opportunistic nature of the sample collection method, sampling of the same individual may have occurred. Multiple replicates from the same individual could lead to inaccuracies such as underestimation of genetic diversity (Bashalkhanov *et al.* 2009; Jackson *et al.* 2016; Schultz *et al.* 2021). However, as I filtered the samples both temporally and spatially, combining this with molecular sexing data, the number of repeated samples from individuals is likely to be minimal. Overall, this study provides evidence suggesting that the Grenada Dove has low mitochondrial genetic diversity. Given the hypothesis that, shortly after colonisation, the Grenada Dove has existed in a small and geographically isolated population (Chapter 4), this long-term genetic isolation may be an important factor for this species (Clegg, 2010; Peters et al., 2022).

5.5.2 Population comparison

This study provides evidence of genetic differences between the Grenada Dove from two different sites: Perseverance in the West and Mt Hartman Estate in the Southwest of the island. One haplotype (Haplotype A: Mt Hartman=100%, Perseverance=25%) was shared between both locations whereas the other haplotype identified (Haplotype B: Mt Hartman 0%, Perseverance=75%) was unique to Perseverance. Higher haplotype and nucleotide diversities were observed within the samples at Perseverance (n=8) compared to Mt Hartman (n=15), which had no genetic variation despite a larger sample size and larger population (Rusk, 2009, 2017). This highlights the important role of the dry forest at Perseverance as the location of the highest genetic diversity for this species, particularly as Perseverance is at the smaller of the two populations (Rusk, 2017). Overall, these data indicate that there is genetic divergence between geographically sub-divided populations (F_{ST} = 0.71). However

caution is recommended as the nucleotide difference among haplotypes is just one polymorphic variation which potentially affects the interpretation through possible overestimation of the divergence (Bhatia *et al.* 2013). Both the haplotype unique to Perseverance and the genetic divergence between the two localities suggest that these populations may now be isolated from each other. As there was also a haplotype shared between both populations, these data supports the belief that these regions were historically connected and have since become isolated (Rusk, 2017). Population isolation is a threat to this species given its already small numbers (Rusk, 2009) as small isolated populations are at risk of losing genetic diversity over time through stochastic genetic drift (Fraser, 2017). If, as the results indicate, there is little to no gene flow between the two populations, this reduces potential for mate choice and increases the probability of mating between related individuals (Jensen *et al.* 2007; Price *et al.* 2015). Such low gene flow increases the risk of extinction to small populations whose genetic diversity can erode as a result of inbreeding (Kahilainen *et al.* 2014). Future genetic studies should include nuclear regions to discern the extent to which the Grenada Dove populations are isolated, and help assess levels of inbreeding (Ewing *et al.* 2008; Hagen *et al.* 2011; White *et al.* 2014). For example, Rudnick *et al.* (2005) used microsatellite loci obtained from naturally shed feathers to assess relatedness among mated pairs and characterise annual turnover in an adult breeding population of Endangered Eastern Imperial Eagle *Aquila heliacal* in Kazakhstan. Furthermore, genomic data would provide an understanding this species adaptive potential, however, this would require access to higher quality samples (Ryan, 2021; Chapter 1).

5.5.3 Molecular sexing

Of the samples which could accurately be assigned a sex, the sample set comprised 18 males and 5 females. The samples assigned as female consisted of only Haplotype A. The samples assigned as male consisted of 61% Haplotype A and 28% Haplotype B. All samples from females were collected at Mt Hartman whereas 66.6% of the male samples were collected from Mt Hartman and 33.33% from Perseverance. Whilst this result may be indicative of a male skewed sex ratio, caution is needed because of the non-invasive, opportunistic sample collection method. Due to the paucity of

information regarding the breeding ecology of this species, such as the effects of age, sex, climate and breeding cycles on moult (Brown *et al.* 2002; Leeson and Walsh 2007; Kiat *et al.* 2019), I was unable to interpret whether bias could have been generated by the sample collection method. Future studies could consider sampling across the whole year to mitigate against biases if factors such as breeding season influence male moult (Leeson and Walsh 2007; Kiat *et al.* 2019). A 1:1 adult sex ratio (ASR) has often been assumed for the Grenada Dove even though it has been acknowledged that there is potential for a male-sex bias (Bolton *et al.* 2015; Rivera-Milán *et al.* 2015). A male skew is not uncommon in pigeons and doves as evidenced by the Pink Pigeon (Bunbury, 2006), Scaled Pigeon *Patagioenas speciose* and Plain-breasted Ground Dove *Columbina minuta* (Bosque & Pacheco, 2019). Skewed sex ratios may not be the result of unequal production of the sexes at birth but could be the result of differential adult survival rates where one sex is at higher risk of predator based mortality or more susceptible to parasites and disease (Székely *et al.* 2014; Bosque and Pacheco 2019). Many pigeons show bi-parental care, with females often incubating and brooding at night, which makes them more vulnerable to nocturnal predators such as rats and manicou (Baptista et al., 2020; Donald, 2007; Gibbs et al., 2010; Twyman & Hayslette, 2007), differential predator rates, therefore, could potentially be a major consideration for this species. There have been number studies on ASR in bird populations which have revealed, for example, that a male skew is frequently seen in threatened species (Donald, 2007) as well as species with small ranges and island species (Venables & Brooke, 2015). The majority of studies, however, have been carried out on species in temperate latitudes and so sex ratio information for birds at tropical latitudes is largely unknown (Bosque & Pacheco, 2019). The results collected in this study reflect this suspected male skew which, if correct, could mean that the reported population size for this species may be lower than current estimates, which are extrapolated from data on calling males (Bolton *et al.* 2015). If accurate, the low maternal genetic diversity in this data could be influenced by this male-sex bias (Houston *et al.* 2015). Carrying out genetic diversity assessment using nuclear markers, which are inherited from both parents, would avoid these issues, however this was not possible in this study because of the type and degraded nature of the samples (Peters *et al.* 2019). ASRs are a key component in understanding species'

demography and biology (Donald 2007; Székely *et al.* 2014; Bosque and Pacheco 2019) and therefore further investigation into the sex ratio, such as the banding programme proposed in Bolton *et al.* (2015), could be critical for the survival of this Critically Endangered species.

5.5.4 Conservation Implications

To conserve species with fragmented or isolated populations, an understanding of genetic diversity is recommended before undertaking any management interventions (vanDyke 2008; Frankham *et al.* 2017). The data from this study revealed unique genetic variation at one locality (Perseverance) suggesting additional habitat protection at this site is important for conserving genetic diversity in this species (Tarr & Fleischer, 1999). Re-introduction of the Grenada Dove to dry forest in its northern historic range, using individuals from both Mt Hartman and Perseverance, could also be beneficial as geographically separate populations are not only important for promoting genetic diversity (Gregory *et al.* 2012; du Plessis *et al.* 2019; Major *et al.* 2021) but also in safeguarding against natural disasters such as hurricanes. Given recent habitat loss on Grenada, availability of suitable habitat on the island for establishing new populations is limited and would likely result in additional isolated populations (Rusk *et al.* 2008; Prugh *et al.* 2008). Additional separate populations can be a useful conservation approach, serving as insurance populations for species with small numbers and limited occurrence, should other populations be threatened (Major *et al.* 2021). Establishing new populations using a small number of founder individuals is a viable approach when considering species of conservation concern (Grueber *et al.* 2017). This is not uncommon in island species and populations have been known to successfully recover from a very small number of individuals (Copsey *et al.* 2018) for example the Pink Pigeon went from 9 or 10 individuals in 1990 to 446 in 2011 (Swinnerton *et al.* 2004), the Echo Parakeet went from as low as 12 individuals in the 1990s to more than 500 in 2010 (Raisin *et al.* 2012) and the Rodrigues Warbler *Acrocephalus rodericanus* started with approximately 17 individuals in 1979 to more than 3000 in 2010 (Showler *et al.* 2002; Copsey *et al.* 2018). Additional isolated populations may be useful for rescue measures and to protect against localised extinction (Grant *et al.* 2001; du Plessis *et al.* 2019; Frankham *et al.* 2019), however, regular assessment of

genetic variability is essential to monitor whether new populations are successfully established (Gregory *et al.* 2012).

I provide evidence of population isolation for the Grenada Dove. Conventionally, recommendations to increase gene flow between isolated populations would be through habitat connectivity (Lindsay *et al.* 2008; Frankham *et al.* 2019). However, this is unrealistic in this case due to the expansive human development which separates the two sites. Augmentation of gene flow may be more easily facilitated on this occasion by translocation of individuals with Haplotype B between the populations. Despite evidence of low genetic diversity at each population such genetic rescue measures were successfully used on isolated populations of an island endemic species, the South Island Robin (Heber *et al.* 2012). Heber *et al.* (2012) used highly inbred populations as donors to rescue two isolated populations. Translocations of 31 female robins led to an increase in heterozygosity, immunocompetence and juvenile survival in individuals crossed between the two populations in comparison to the those crossed within each population. I therefore recommend the implementation of genetic rescue measures with the aim of augmenting genetic diversity at Mt Hartman and reducing the risk of extinction at this site (Frankham *et al.* 2019).

5.6 Conclusion

This study has shown low mitochondrial genetic diversity, evidence for genetically isolated populations, and a potentially male skewed sex ratio in the Grenada Dove. Two haplotypes were identified, the most common haplotype was observed at both sites and the other was unique to Perseverance. This suggests historical connectivity and subsequent isolation of these two populations. These data suggest that the population at Perseverance is the most genetically diverse of the two highlighting the importance of this forest in maintaining the genetic diversity of this species. In light of the findings of this study, I recommend habitat protection, establishing a new population, and augmentation of gene flow by translocation in order to increase genetic resilience and diversity, with the ultimate aim of securing the long-term survival of this Critically Endangered species.

CHAPTER 6 Population Viability Analysis

Photo by Author

Chapter 6: Assessing threats and conservation action using Population Viability Analysis for the Critically Endangered Grenada Dove *Leptotila wellsi*

6.1 Abstract

Accurate estimation of extinction risk is crucial to preventing further declines of wild populations and is a useful conservation tool when deciding on appropriate management actions. The Critically Endangered Grenada Dove is at risk from habitat loss and de-forestation, disease, predation by invasive species, a male-skew sex bias and natural disasters. A variety of conservation management strategies have been recommended in the 2008 Species Recovery Plan for the Grenada Dove such as establishing a new population, predator control and a captive breeding programme. Here I used original data, along with published long-term population count data, and data from closely related species to conduct population viability analysis (PVA) generating 50-year simulations for the two populations of Grenada Dove to evaluate extinction risk for this species. This study found that the probability of extinction in the West (PE: 56.8%) was substantially higher than the probability of extinction in the Southwest (PE: 7.6%). The projections obtained under threat scenarios showed significantly decreased population growth rates compared to the standard baseline model and management scenarios increased population growth rates significantly. This PVA has identified that loss of forest habitat to commercial development is likely to have the biggest impact on extant Grenada Dove populations, that head-starting is likely be the most effective conservation strategy and highlights important information on survival and mortality rates which are urgently needed for conserving this Critically Endangered species.

6.2 Introduction

Global loss of biodiversity is a central concern in conservation biology, and accurate estimation of extinction risk is crucial in preventing further declines of wild populations (Reed *et al.* 2003; Olah *et al.* 2016). Deciding on appropriate conservation actions can however, be hindered by a poor understanding of the causes of population decline (Olah *et al.* 2016). Population viability analysis (PVA) is a modelling technique which uses demographic data to evaluate threats, identify effective management strategies and ultimately estimate extinction risk (Chaudhary & Oli, 2020; Lacy, 2019). PVAs have been used to investigate, for example, the impacts of land use change and fragmented ecosystems (Winton *et al.* 2020), the sensitivity of wild populations at risk of harvesting for international trade (Valle *et al.* 2018) and the effects of proposed management strategies (Aresu *et al.* 2021). Evidence for the most effective and achievable management actions resulting from PVAs can be incorporated when developing species recovery plans (Chaudhary & Oli, 2020). As such, PVAs are essential conservation tools for Critically Endangered species, particularly those with small, fragmented populations, which are at increased risk of extinction (Chaudhary & Oli, 2020).

A lack of detailed ecological and demographic information is a risk for any species, particularly those that are Critically Endangered. Extant populations of the little-known, Critically Endangered Grenada Dove are small and isolated (Bolton *et al.* 2015). These populations are threatened by natural disaster, such as hurricanes (Rusk, 2017), habitat destruction and degradation of dry forest (Rusk 2017; Nelson *et al.* 2018) and predation by invasive species, particularly Mongooses, which are thought to predate eggs and fledglings (Rusk 2017; Lees *et al.* 2022). Additional threats include commercial development; with a proposed tourist resort on land at Mt Hartman Estate which forms a large proportion of habitat for the population in the Southwest (Rusk 2008; Rosenberg 2018; Lees *et al.* 2022), and the population in the West being at risk of fires from a nearby landfill site (Rusk *et al.* 2008). Furthermore, both a male skewed sex ratio - frequently observed in pigeons and threatened bird species (Donald 2007; Chapter 5), and disease spread through shared water sources - water-borne

trichomonosis and mosquito-carried avian malaria (Bolton *et al.* 2015) represent potential threats; however, their nature and extent are yet to be determined (Rivera-Milán *et al.* 2015; Lees *et al.* 2022).

A recovery plan has been drafted in order to prevent further decline and help ensure the survival of this endemic species (Rusk *et al.* 2008). The plan includes ecotourism and community outreach programmes which have been established to educate and build support and awareness for Grenada's national bird and the threats it faces (Rusk *et al.* 2008; BirdLife International 2021a). A predator control programme has successfully trapped Mongooses, Rats, Cats and Opossum in Grenada Dove habitat which are all thought to predate this species (Rusk, 2017). The Grenada Dove is also a legally protected species on the island, providing protection against hunting and egg-collecting, and two of the five Important Bird Areas (IBAs) in Grenada - Mt Hartman national park and Perseverance dove sanctuary – are protected Grenada Dove habitat (Rusk 2009; Bolton *et al.* 2015).

Future conservation actions proposed for the Grenada Dove include conducting comprehensive ecological and behavioural research, additional habitat protection and restoration, providing alternatives to standing water sources to prevent spread of disease and establishment of long-term monitoring programmes (Bolton *et al.* 2015; Rivera-Milán *et al.* 2015; BirdLife International 2021a). Consideration of captive breeding/artificial propagation programmes and the establishment of new populations are also proposed conservation actions (Rusk *et al.* 2008; BirdLife International 2021a), with other threatened island endemic species – including Pink Pigeon, Takahē, Saint Lucia Amazon *Amazona versicolor* and Black Stilt *Himantopus novaezealandiae* – recovering via captive breeding programmes with small founder numbers (Reed *et al.* 1993; Jeggo *et al.* 2000; Grueber and Jamieson 2011; Jackson *et al.* 2022). Establishing new populations requires identifying and restoring suitable habitat as release sites; (BirdLife International, 2021b; Rusk, 1998) this method has been successfully implemented for other bird species such as the Brown Teal *Anas chlorotis* where birds were taken from two wild populations and a captive population to establish four new populations (Bowker-Wright *et al.* 2012). Although not proposed in the Grenada Dove recovery plan, head-starting is a conservation method whereby eggs and young are removed from the wild, hand-reared in

captivity and released to augment wild populations (Cunninghame et al., 2015; Gerlach, 2001; Jones, 2002; Jones & Merton, 2012). This strategy has been successfully used to increase survival during a species' most vulnerable life stages (Collins *et al.* 2016; Cunninghame *et al.* 2015; Ross *et al.* 2021), and could be used to mitigate the threat of predation for the Grenada Dove.

Here I investigated the influence of stochastic demographic events and conservation management approaches over a 50-year period for two small, isolated populations of the Critically Endangered Grenada Dove. A PVA, using Vortex software (Lacy & Pollak, 2021), was conducted to a) develop a baseline model to ascertain the population trajectory based on current conditions; b) model threat scenarios to establish their effects on population persistence and c) simulate population trajectories using a variety of different conservation management strategies. This information was then used to determine which threats pose the largest risk to survival and suggest optimal management strategies to inform conservation decisions for this Critically Endangered species.

6.3 Methods

The development and implementation of this PVA was carried out using Vortex 10 version 10.5.5.0 (Lacy & Pollak, 2021). Vortex is the most commonly used software package for modelling population viability (Chaudhary & Oli, 2020). Population models were simulated with 500 iterations across a 50-year period which represents more than 10 generations of Grenada Dove, where generation length is approximately 4.2 years as calculated by the IUCN generation length calculator (BirdLife International, 2021b). A two-population projection was modelled to reflect the two extant isolated populations of the Grenada Dove (Rusk 2017; Chapter 5). The models do not incorporate environmental variables and genetic management, due to the lack of appropriate data, both of which would have an effect on vital rates and growth rates over time (Tenhumberg *et al.* 2004). A quasi-extinction threshold where extinction was defined as less than 20 individuals was used (Medina-Miranda *et al.* 2012; Morris & Doak, 2002). Therefore, the probability of extinction was calculated based on populations falling below this threshold as these very small populations are considered unlikely to be viable.

6.3.1 Standard Model

I parametrised the standard 'baseline' model (Table 6.1) with original data generated by this thesis, previously published data and expert knowledge on this species. As some parameters of the Grenada Dove have yet to be determined, input parameters were obtained from discussions with Grenada Dove experts based on circumstantial and preliminary evidence and published data on closely related species, a process commonly used when data are not available for PVAs (Aresu *et al.* 2021; Bruford *et al.* 1991; Valle *et al.* 2018; Winton *et al.* 2020). The standard model was built to reflect an approximation of the current population trend observed in population count data (Rusk, 2017). As inbreeding is assumed in small populations, particularly those with evidence of low genetic diversity (Frankham *et al.* 2010), inbreeding depression was incorporated into the model. Hurricanes and general habitat loss were incorporated into all Grenada Dove population models as 'catastrophes'. Hurricanes occur approximately once every 50 years (frequency 2%) in Grenada. When using population counts pre- and post-hurricane (Rusk, 2017) there was an 80% decline in the West (survival 0.2) and a 30% decline in the Southwest (survival 0.7). While it has been documented that changes in breeding behaviour of Grenada Dove were observed post Hurricane Ivan in 2004 (Rusk, 2017), the effects of hurricanes on reproduction have not yet been quantified and as such I assumed reproduction was unaffected. There has been a 4.2% decrease in tree cover between 2001 and 2020 in Saint George's, Grenada (Global Forest Watch, 2020). Therefore, habitat loss was modelled with a 0.21% frequency with a <1% negative impact on survival (survival 0.99) assuming dove loss was proportional to habitat loss over a 50-year period.

Table 6.1: Vortex parameter inputs and sources for the standard model for populations of the Grenada Dove.

Parameter	Value(s)	Source
Scenario settings		
Total N < Critical size	20	Medina-Miranda *et al.*(2012); Morris & Doak (2002)
Number of populations	2	Rusk (2017)
Species Description		
Inbreeding Depression	Checked	Original data, Chapter 5
Lethal Equivalents	6.29	O'Grady *et al.*(2006)
Percent due to recessive lethal alleles	50	O'Grady *et al.*(2006)
Dispersal		
Dispersal Among Populations	No dispersal	Original data, Chapter 5
Reproductive System		
Reproductive System	Monogamous	Winkler *et al.*(2020)
Age of First Reproduction	1	Pedro de Magalhães (2021)
Maximum Age of Reproduction	13	Rob Martin, Grenada Dove BirdLife International Data
Maximum lifespan	13	Rob Martin, Grenada Dove BirdLife International Data
Maximum Number of Broods per Year	2	Original data, Chapter 4; Peters *et al.* (2022)
Maximum Number of Progeny per Brood	2	Original data, Chapter 4; Peters *et al.* (2022)
Sex Ratio at Birth	1:1	Albeshr (2016)
Reproductive Rates		
% Adult Females Breeding	100%	*NA*
EV in % Breeding	10%	Bruford *et al.*(1991)
Distribution of broods per year 0 broods	29.7*	Original data, Chapter 4; Peters *et al.* (2022)
Distribution of broods per year 1 broods	41.2*	Original data calculated from Chapter 4; Peters *et al.* (2022)
Distribution of broods per year 2 broods	29.1*	Original data calculated from Chapter 4 Peters *et al.* (2022)
Offspring distribution: 1 brood	41.4†	Original data calculated from Rusk (2017)
Offspring distribution: 2 brood	58.8†	Original data calculated from Rusk (2017)
Mortality Rates		
Mortality from age 0-1	55.9	Giese *et al.* (2018)
SD in 0-1 mortality due to EV	5	Bruford *et al.*(1991)
Annual mortality after 1	33.8	Giese *et al.* (2018)
SD in mortality after 1	5	Bruford *et al.*(1991)
Catastrophes		
Catastrophe Label	Hurricane	*NA*
Frequency and extent of occurrence: Frequency %:	0.02	National Hurricane Center (2022)
Severity (proportion of normal values): Reproduction	1	*NA*
Severity (proportion of normal values): Survival	Perseverance: 0.2‡ Mt Hartman: 0.7	Original data calculated from Rusk (2017)
Catastrophe Label	Habitat loss	*NA*
Frequency and extent of occurrence: Frequency %:	0.21	Global Forest Watch (2020)
Severity (proportion of normal values): Reproduction	1	*NA*
Severity (proportion of normal values): Survival	0.99	Original data calculated from Global Forest Watch (2020)
Initial Population Size		
Initial Population Size	West: 45 ^ Southwest: 115	Original data calculated from Rivera-Milán *et al.* (2015); Rusk (2017)
Carrying Capacity		

* Calculated using random Poisson distribution (truncated Poisson) using 1.4 clutches per year value generated in Chapter 4; (Peters et al 2022).
† Values allowed replication of population trend calculated from long-term population count data (Rusk, 2017)
‡Values calculated from long-term population count data before and after hurricane Ivan in 2004 (Rusk, 2017)
^ Values generated from long-term population count data (Rusk, 2017) and distance sampling survey and abundance estimation (Rivera-Milán *et al.* 2015)

6.3.2 Sensitivity Analysis

Sensitivity analysis was performed, using a perturbation approach (single factor) where parameters were varied by ±5%, ±15%, ±25% and ±50% (see Appendix XI), as per method used by Aresu *et al.* (2021). Sensitivity tests are used to understand parameter uncertainty and parameter effect on model outcomes (Aresu *et al.* 2021; Lacy, 2019). Mortality and reproductive rates parameters were investigated including juvenile and adult mortality and percentage of breeding females. Catastrophe parameters that were built into the standard model including frequency and severity of hurricanes and habitat loss were also examined. Each scenario was run for 500 iterations and 50 years and variation across the simulated parameter values for probability of extinction, population growth rates and mean size of the extant population (N-extant) for the two populations of Grenada Dove were compared.

6.3.3 Threat Scenarios

6.3.3.1 Loss of Mt Hartman

The proposed development of a tourist resort would mean a loss of a significant proportion of Mt Hartman estate which poses a threat to the largest breeding population of Grenada Dove (BirdLife International, 2021b; Rosenberg, 2018; Rusk, 2010b). To model this, a catastrophe was set up to affect only the Southwest population, which is where Mt Hartman is situated. As I wanted to model the proposed development going ahead, I modelled a 100% chance that this event would occur. As only doves in the unprotected area of Mt Hartman would be affected in the Southwest, numbers were adjusted to approximate how many doves live in this area. Based on population count data (Rusk, 2017), I approximate a little more than 86% of the Southwest population are found at Mt Hartman Estate. Of the doves at Mt Hartman Estate, it is also estimated that 52% (approx. 60) are found at Mt Hartman national park (Rusk, 2009), with the assumption that 35% (approx. 40) are in the unprotected area. This leaves the remaining 13% (approx. 15) in the Southwest in areas outside of Mt Hartman Estate. Assuming only doves in the unprotected area of Mt Hartman estate (35%) are affected and

doves in the national park and in the surrounding area at the Southwest survive (65%) a 35% negative impact on survival (survival 0.65) was modelled.

6.3.3.2 Disease

It is thought that water-borne disease spread of *trichomonosis* and mosquito-carried avian malaria could have health implications affecting reproduction and survival of Grenada Dove (BirdLife International, 2021b; Bolton *et al.* 2015). However, there are no data available on the types or impact of disease on Grenada Dove populations to date, therefore data on avian epidemics was used (Bruford *et al.* 1991) to model this threat. Models included a 1% frequency of disease outbreak, with a 10% negative impact on reproduction (reproduction 0.9) and a 50% negative impact on survival (survival 0.5).

6.3.3.3 Sex ratio

Adult Sex Ratios (ASRs) in threatened bird species are often skewed and it has been acknowledged that there is the potential for a male-sex bias for the Grenada Dove (BirdLife International, 2021b; Bolton *et al.* 2015; Donald, 2007). In Chapter 5, molecular sexing was conducted on samples obtained for this study and the sample set was found to contain more males than females which may be indicative of a male biased sex ratio for this species. Therefore, a sex bias scenario was modelled. The sex ratio was kept even at birth as there is no evidence to suggest that sex ratio deviates from parity at this stage. A male skew has been modelled at fledging age, as is observed in other endangered pigeon species (Albeshr, 2016). A 0.7 sex ratio was used as reported for Critically Endangered wild bird species (Donald, 2007). As such, mortality rates from age 0-1 was adjusted to 80.8% for females to reflect a male-sex bias.

6.3.4 Management scenarios

6.3.4.1 Supplementation

Captive breeding/artificial propagation (BirdLife International, 2021b) and establishment of new populations are proposed conservation actions proposed for the Grenada Dove (Rusk *et al.* 2008).

Supplementation of birds into the wild from a captive stock was modelled where 10 birds were removed from existing populations gradually over a 10-year period (Swinnerton, 2001; Young *et al.* 2012). Birds were harvested from both populations to maximise genetic diversity (see Chapter 5). Assuming a 10-year captive breeding programme, populations were supplemented with low (4), medium (12), and high (20) numbers of individuals (equal numbers of females and males) aged 0-1 every year from year 10 for the next 30 years, based on length of time before first release (10 years), release numbers (groups of 4-8) and age (juveniles) of pink pigeon captive breeding programme (Bruford *et al.* 1991; Swinnerton, 2001). Vortex assumes individuals being supplemented are unrelated to recipient populations (Lacy & Pollak, 2021).

6.3.4.2 Head-starting

Head-starting conservation strategies often work on the assumption that by removing eggs from a breeding pair early, the pair will re-lay with the second clutch being left to be reared in the wild (Simpson, 2020). This scenario was modelled based primarily on that of a head-starting programme of an Critically Endangered island bird, the Mangrove Finch *Geospiza heliobates* (Cunninghame *et al.* 2015). Head-starting was represented by increasing the clutch sizes of a proportion of the population to represent the 'head-started' clutch surviving to release, hence, an increase in juvenile individuals were supplemented into each population over a 10-year timespan. A 0.9 survival rate was used based on chicks released into the wild having 90% (0.9) survival post release (Swinnerton, 2001). Birds were head-started in low – 5%, medium – 10% (Cunninghame *et al.* 2015) and high – 15% numbers of equal sexes of juvenile birds

6.3.4.3 Establishing a new population

Establishing a new population was modelled both with and without a captive population. As availability of suitable habitat for re-introductions is important (Black 1991; Dolman *et al.* 2015) and available habitat is limited on Grenada, the establishment of just one population was modelled. Hypothetically, this population would be in the dry forest on the North of Grenada which was part of

the Grenada Dove's historical range and is geographically separate to the populations in the West and Southwest thus acting as a reserve population mitigating the risk of hurricanes (Baptista *et al.* 2020; BirdLife International, 2021b; Bond, 1973; Lack & Lack, 1973). Generally, establishing new populations occurs as a result of a captive breeding programme, however, in the Grenada Dove Species Recovery Plan establishing new populations is proposed without mention of a captive breeding programme (Rusk *et al.* 2008) and in the IUCN Red List 'Conservation Actions Proposed' it is mentioned prior to consideration of a captive breeding programme - "*Establish two new subpopulations (Rusk et al. 1998) and consider establishing a captive breeding population*" (BirdLife International, 2021b).

Therefore, modelled birds were harvested from the two wild populations (to maximise genetic diversity – see Chapter 5). As removing large numbers of birds may risk extant populations (Dolman *et al.* 2015) birds were harvested proportionally from the Southwest and West populations to reflect differing population sizes. Birds were translocated to a new population over a 5 year period, based on release of pink pigeon at three locations where birds were translocated to each site throughout a 4.2 year period on average (Swinnerton, 2001). The new population was established using 1%, 3% and 5% of the birds from the Southwest and West populations. Birds were translocated in equal numbers of sexes and equal numbers of adults and juveniles to ensure that both breeding birds and birds which would not affect territorial behaviours were released (Swinnerton, 2001).

Establishing a new population using a captive breeding programme was also modelled. The captive breeding programme was modelled as outlined above in the supplementation section (harvesting 10 birds over 10 years). Birds were then translocated in low – 10 (Elzinga *et al.* 2020) , medium - 15 and high – 20 (Collar, 2020) numbers as described above (over a 5 year period in equal numbers of sex and adults and juveniles).

6.3.5 Data Analysis

Stochastic growth rates of different threats and management scenarios were compared to the standard baseline model using analysis of variance (ANOVA) (Long & Teetor, 2019) performed in R

version 4.1.2 (R Core Team, 2021). The ANOVA was performed by fitting a linear model with population growth rate as the response and the scenario as the predictor, with the standard model set as the reference factor.

6.4 Results

6.4.1 Standard Model

Projected population trend predictions for the next 50 years (Figure 6.1) approximately reflect trends observed in population count data between 1987 and 2007 (Rusk, 2017), with a growth rate of -0.0024 (95% CI [-0.0026, -0.0022]) for model data and a growth rate of 0.0275 (95% CI [-0.0043, 0.0593]) observed data. The population growth rates in the West showed a decline (-0.0085 (95% CI[-0.018, -0.027])) whereas the population in the Southwest is relatively stable (-0.0050 (95% CI [-0.013,-0.018])) (Table 6.2). The probability of extinction in the West was substantially higher at 56.8% with the mean time to extinction of 25.3 years in contrast to the probability of extinction in the Southwest which was 7.6% with the mean time to extinction of 38.9 years.

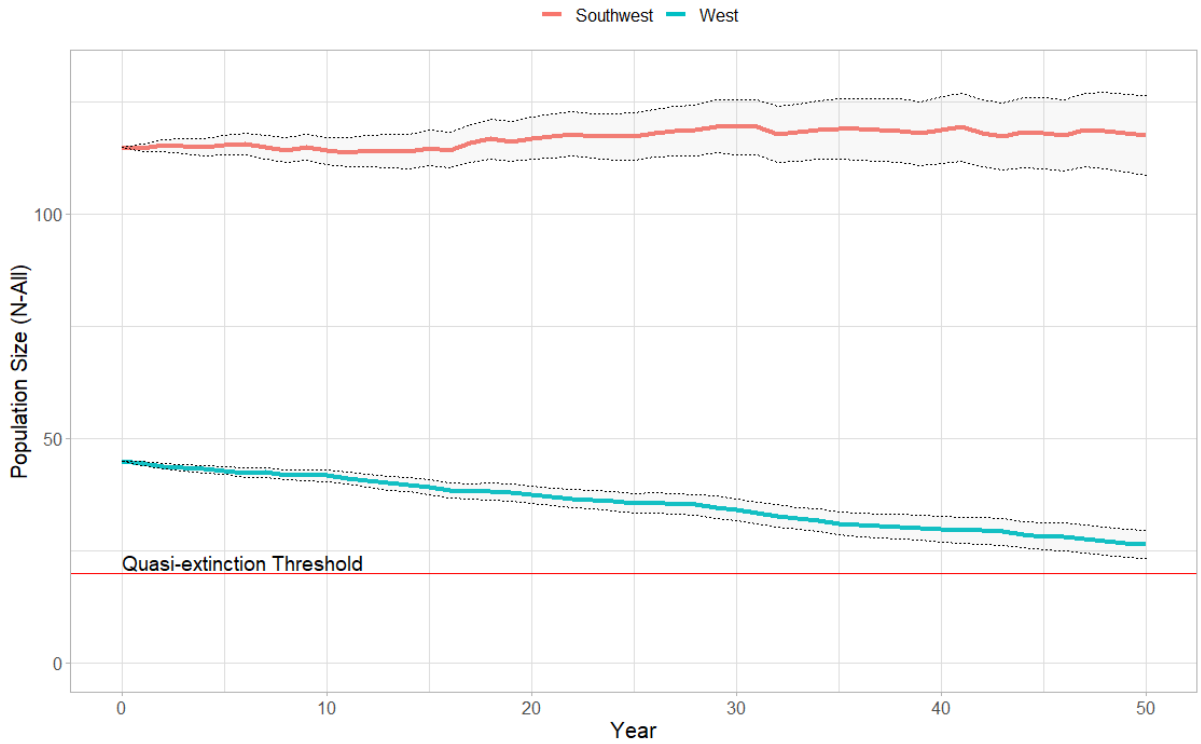

Figure 6.1: Predicted 50-year trends for the standard baseline model for both Southwest and West populations with 95% CI. Quasi-extinction threshold line in red intercepting the y-axis at 20 individuals.

Table 6.2. Stochastic results for different scenarios simulated (50 years, 500 iterations) in a PVA for the Grenada Dove. Stoch-r: stochastic growth rates; PE: proportion in which the population went extinct; N-extant: mean population size, only for those remaining extant; N-all: mean population size, across all population extant or extinct; Mean TE: of iterations that suffer extinctions

Scenario	Population	stoch-r	SD(r)	PE	N-extant	SD(N-ext)	N-all	SD(N-all)	meanTE
Standard Model	Southwest	-0.0050	0.0960	0.0760	126.57	99.63	117.50	100.88	38.9
Standard Model	West	-0.0085	0.1111	0.5680	54.63	39.04	26.51	35.82	25.3
Threats									
Loss of Mt Hartman	West	-0.0086	0.1127	0.5740	55.67	46.97	26.66	39.82	26.1
Loss of Mt Hartman	Southwest	-0.4253	0.1059	1.0000	0.00	0.00	0.00	0.00	4.6
Disease	Southwest	-0.0119	0.1204	0.1720	104.93	99.60	88.12	97.89	35.6
Disease	West	-0.0129	0.1239	0.7500	53.61	41.99	16.23	30.49	23.1
Sex Bias	Southwest	-0.1616	0.1184	1.0000	0.00	0.00	0.00	0.00	10.9
Sex Bias	West	-0.1289	0.1233	1.0000	0.00	0.00	0.00	0.00	6.2
Management Strategies									
Suppl High	Southwest	0.0167	0.0921	0.0000	304.23	175.91	304.23	175.91	0.0
Suppl High	West	0.0285	0.1056	0.0000	206.20	110.85	206.20	110.85	7.8
Suppl Medium	Southwest	0.0105	0.0923	0.0000	225.89	134.08	225.89	134.08	0.0
Suppl Medium	West	0.0183	0.1053	0.0100	125.40	72.23	124.29	72.73	11.4
Suppl Low	Southwest	0.0002	0.0942	0.0100	144.88	96.49	143.57	96.89	41.6
Suppl Low	West	0.0009	0.1123	0.2400	61.41	41.63	48.98	42.61	20.2
New Pop High	North	0.0091	0.1313	0.6200	48.70	28.93	21.51	28.19	1.0
New Pop High	Southwest	-0.0090	0.0994	0.1040	111.33	94.04	100.77	94.28	38.0
New Pop High	West	-0.0122	0.1197	0.6700	50.51	41.42	19.49	32.59	20.8
New Pop Medium	North	0.0168	0.1302	0.8500	37.52	20.16	7.98	15.37	1.0
New Pop Medium	Southwest	-0.0066	0.0972	0.0820	118.64	90.76	109.77	91.91	39.7
New Pop Medium	West	-0.0121	0.1172	0.6540	48.61	35.13	19.66	29.90	21.7
New Pop Low	North	0.0472	0.1371	0.9960	33.00	12.73	0.39	2.74	1.0
New Pop Low	Southwest	-0.0051	0.0960	0.0700	124.14	91.10	116.22	92.49	39.4
New Pop Low	West	-0.0091	0.1128	0.6100	57.64	42.89	25.14	37.56	24.4
New Pop CB High	North	0.0354	0.1544	0.0160	130.73	87.10	128.89	87.60	1.0
New Pop CB High	Southwest	-0.0060	0.0965	0.0680	121.39	92.95	113.80	94.06	38.7
New Pop CB High	West	-0.0114	0.1157	0.6560	50.02	35.34	20.37	30.28	22.3
New Pop CB Medium	North	0.0238	0.1323	0.0660	97.11	67.13	91.45	68.30	1.0
New Pop CB Medium	Southwest	-0.0061	0.0955	0.0740	120.00	90.71	111.90	91.90	39.3
New Pop CB Medium	West	-0.0109	0.1163	0.6400	52.03	39.87	21.29	33.52	21.5
New Pop CB Low	North	0.0170	0.1332	0.2480	62.31	38.82	49.43	40.57	1.0
New Pop CB Low	Southwest	-0.0053	0.0958	0.0700	125.73	99.72	117.46	100.80	38.8
New Pop CB Low	West	-0.0107	0.1164	0.6080	49.47	35.45	21.81	31.74	23.1
Headstarting High	Southwest	0.0273	0.1038	0.0000	562.22	402.50	562.22	402.50	0.0
Headstarting High	West	0.0225	0.1074	0.0280	192.56	158.35	187.44	159.01	37.8
Headstarting Medium	Southwest	0.0173	0.0974	0.0020	345.70	263.98	345.02	264.15	46.0
Headstarting Medium	West	0.0110	0.1064	0.1160	113.75	95.23	101.59	95.65	36.1
Headstarting Low	Southwest	0.0074	0.0941	0.0180	221.83	175.89	218.07	176.50	44.8
Headstarting Low	West	0.0011	0.1072	0.2600	72.16	54.81	55.44	55.05	33.3

6.4.2 Threat scenarios

The projections obtained for all threat scenarios showed that overall these threats decreased population growth rates significantly for the Southwest (F= 17980, df=1996, p=<0.001; Figure 6.2a) and the West (F=1991, df=1996, p=<0.001; Figure 6.2b).

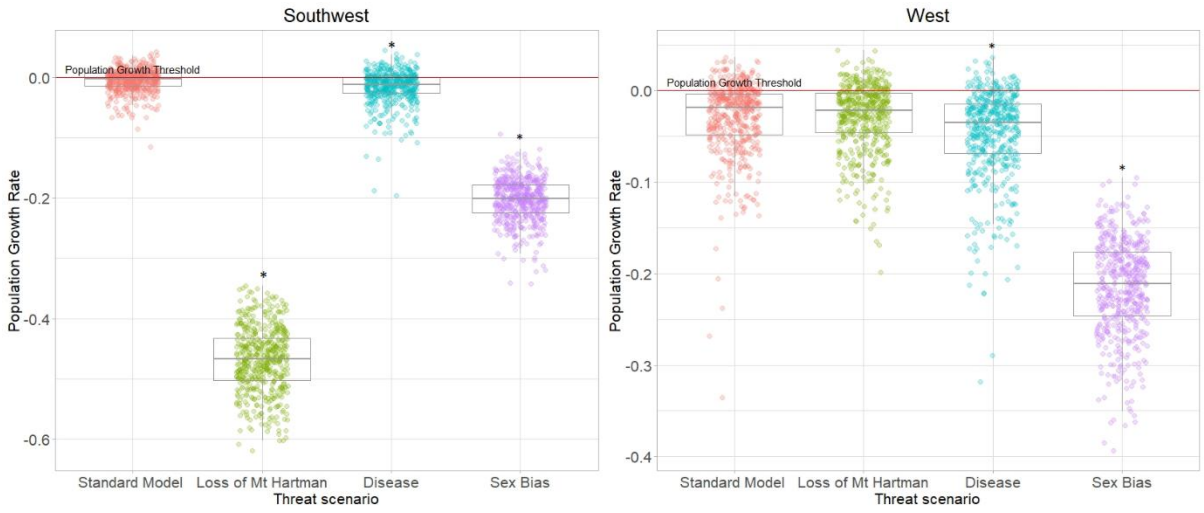

Figure 6.2. Comparative stochastic population growth rates under the different threat scenarios simulated (50 years, 500 iterations) in a PVA for the Grenada Dove for a) Southwest population and b) West population. * indicated significant differences from the standard model: p<0.05.

6.4.2.1 Loss of Mt Hartman

In the Southwest population, the effect of losing a considerable part of Mt Hartman Estate for a proposed commercial development changes the population growth rate at the Southwest to -0.4253 (95% CI [-0.435, -0.860]) which is a significant decrease (t=-202.545, p=<0.001) compared the stable population trend (-0.0050) projected by the standard model. The probability of extinction is increased to 100% (from 7.6%) with a mean time to extinction of 4.6 years. The population in the West is unaffected by any proposed development in the Southwest due to population isolation and hence is not significantly different (t=0.302, p=0.763) to the standard model.

6.4.2.2. Disease

In both populations the introduction of a disease epidemic significantly decreased the population growth rates in comparison to the standard model (West: t= -6.120, p=<0.001; Southwest: t=-4.652, p=<0.001). The probability of extinction in the West increased to 75%, compared to 56.8% projected by the standard model, with the mean time to extinction of 23.1 years. The probability of extinction in the Southwest also increased compared to the standard model (7.6%) to 17.2% with the mean time to extinction of 35.6 years.

6.4.2.3 Sex ratio

The projection obtained when a sex ratio was modelled to reflect a male-skewed sex ratio for Critically Endangered wild bird populations showed a significant decline from the standard model in both populations (West: t= -64.757, p= <0.001; Southwest: t= -86.603, p= <0.001) with a 100% probability of extinction and a mean time to extinction of 6.2 years in the West population and 10.9 years in the Southwest population.

6.4.3 Management scenarios

The projections obtained for all management scenarios showed that overall these scenarios increased population growth rates significantly for the Southwest (F= 193.2, df= 5988, p= <0.001; Figure 6.3a) and the West (F= 278.4, df= 6487, p= <0.001; Figure 6.3b).

6.4.3.1 Supplementation

For all supplementation scenarios the population growth rates turned positive for both populations. Population increases were significantly higher in all standard model comparisons (Southwest – High: t= 26.455, p= <0.001, Medium: t= 19.769, p= <0.001, Low: t =7.224, p= <0.001; West – High: t= 28.635, p= <0.001, Medium: t= 24.299, p= <0.001, Low: t= 13.210, p= <0.001). Supplementation reduced the probability of extinction to 0% under modelled scenario conditions over the next 50 years with the following exceptions: West - PE: Medium = 1%, Low: 24% and Southwest - PE: Low=1%. All other levels of population supplementation removed the probability of extinction in

both populations. In the 10 years after supplementation ended all populations showed positive population growth rates (see Figure 6.4) with the exception of the population growth rate at the West when the low supplementation scenario was modelled.

The optimal release strategy, assuming the lowest number of released birds is the most realistic, appears to be the medium supplementation scenario with 3 male and 3 female juvenile birds being released every year for the next 30 years. The remaining 10 years after supplementation ended shows a positive or stable (see Figure 6.3) population trend in both populations and increased the mean final population size of the extant populations (N-extant: Southwest:225.89, West:125.40).

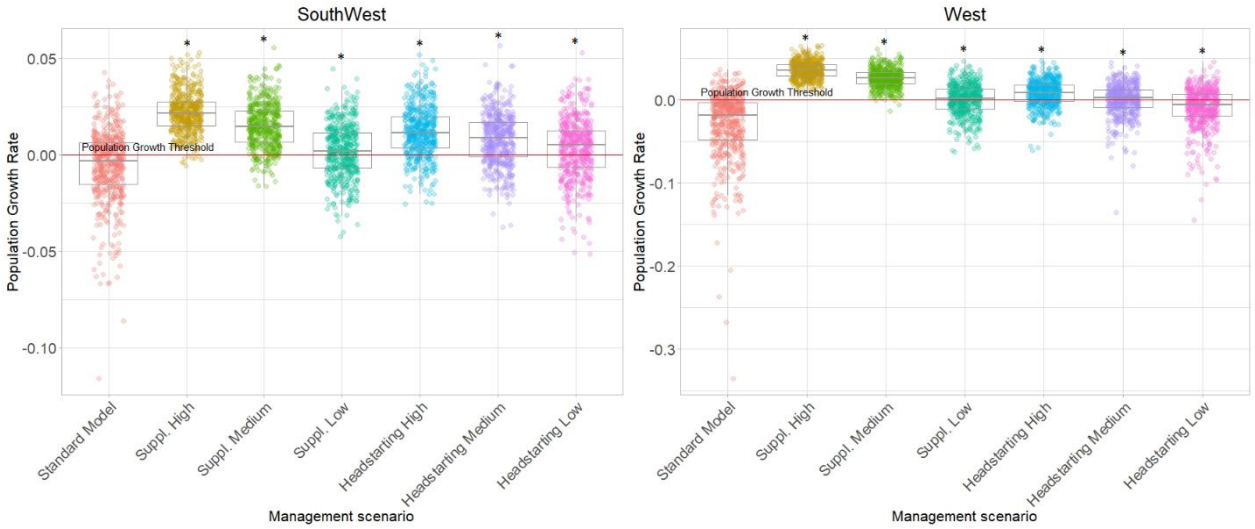

Figure 6.3: Comparative stochastic population growth rates under different management scenarios simulated (50 years, 500 iterations) in a PVA for the Grenada Dove for a) Southwest population and b) West population. Suppl: Supplementation. * indicated significant differences from the standard model: $p<0.05$.

6.4.3.2 Head-starting

Modelling of a 10-year head-starting programme produced a significant increase in population growth rates from negative to positive in both populations at all levels (Southwest – High: $t= 16.736$, $p= <0.001$, Medium: $t= 12.962$, $p= <0.001$, Low: $t= 8.534$, $p= <0.001$; West – High: $t= 16.441$, $p= <0.001$, Medium: $t= 12.930$, $p= <0.001$, Low: $t= 8.890$, $p= <0.001$). The risk of extinction was removed in the

Southwest when head-starting a high number of individuals and was reduced to 0.02% and 1.8% for medium and low levels of head-starting respectively in comparison to the standard model (7.6%). The risk of extinction was not removed in the West but was reduced to 2.8%, 11.6% and 26% for high, medium and low levels of head-starting respectively in comparison to the standard model (56.8%).

The optimal head-starting strategy appears to be the medium supplementation scenario with 10% of juveniles head-started for 10 years. The remaining 40 years after supplementation ended shows a positive population trend in both populations except for the low level of head-starting in the West where the population growth rate turns negative from year 10. The mean final population size of the extant populations (N-extant - Southwest: high= 562.22, medium= 345.70, low= 221.83; West: high= 192.56, medium= 113.75, low= 72.16) all increased compared to that of the standard model.

6.4.3.3 Establishing a new population

When modelling the establishment of a new population using wild birds from extant populations all scenarios failed to produce a self-sustaining population in the North all showing a negative population growth rate after the 5-year translocation period (see Figure 6.4). Both medium and low levels of translocation produced populations below the extinction threshold. High probabilities of extinction were reported for this population at all levels (PE - high: 62%, medium: 85%, low: 99.6%). The projections obtained for the newly established population (North) could not be compared to a baseline standard model as no population currently exist in that area. Establishing a new population using individuals from the two existing populations for establishing a new population significantly decreases population size in comparison to the standard model for both high and medium levels in the West (High: t= -4.905, p= <0.001, Medium: t= -5.303, p= <0.001). For the population in the Southwest this scenario significantly decreases the population size in comparison the standard model when high levels of birds were translocated to the new population (High: t= -2.631, p= 0.00853). Establishing a new population in the North increased the probability of extinction in the two existing populations at all levels, except the low level for the Southwest population.

When modelling the establishment of a new population using a captive breeding programme high and medium levels produced a new population with a positive and stable population growth rate respectively after the 5-year translocation period (see Figure 6.4). All probabilities of extinction were lower than establishing a new population with wild birds from extant populations (PE - high: 1.6%, medium: 6.6%, low: 24.8%). While harvesting birds from extant populations for a captive breeding programme does not affect population growth rate or the probability of extinction for the Southwest it does significantly decrease the population growth rate at the West (high: t= -4.223, p= <0.001, medium: t=-2.555, p= 0.01065, low: t=-3.505, p= <0.001) as well as increase the probability of extinction (PE - high: 65.6%, medium: 64%, low: 60.8%) in comparison to the standard model. Therefore, the optimal strategy specifically for establishing a new population, assuming the establishment of a stable population after management stopped is considered successful, appears to be the medium scenario whereby a 10-year captive breeding programme is used and 15 birds comprising of both juvenile and adult males and females were introduced to a North population for five years. This strategy showed an overall increasing population trend in the North, with a population growth rate of 0.0238 (95% CI [0.012, 0.036]) and an extant population of 97.11. However, none of the modelled scenarios for establishing a new population which produced a stable or increasing population did so without detriment to existing populations.

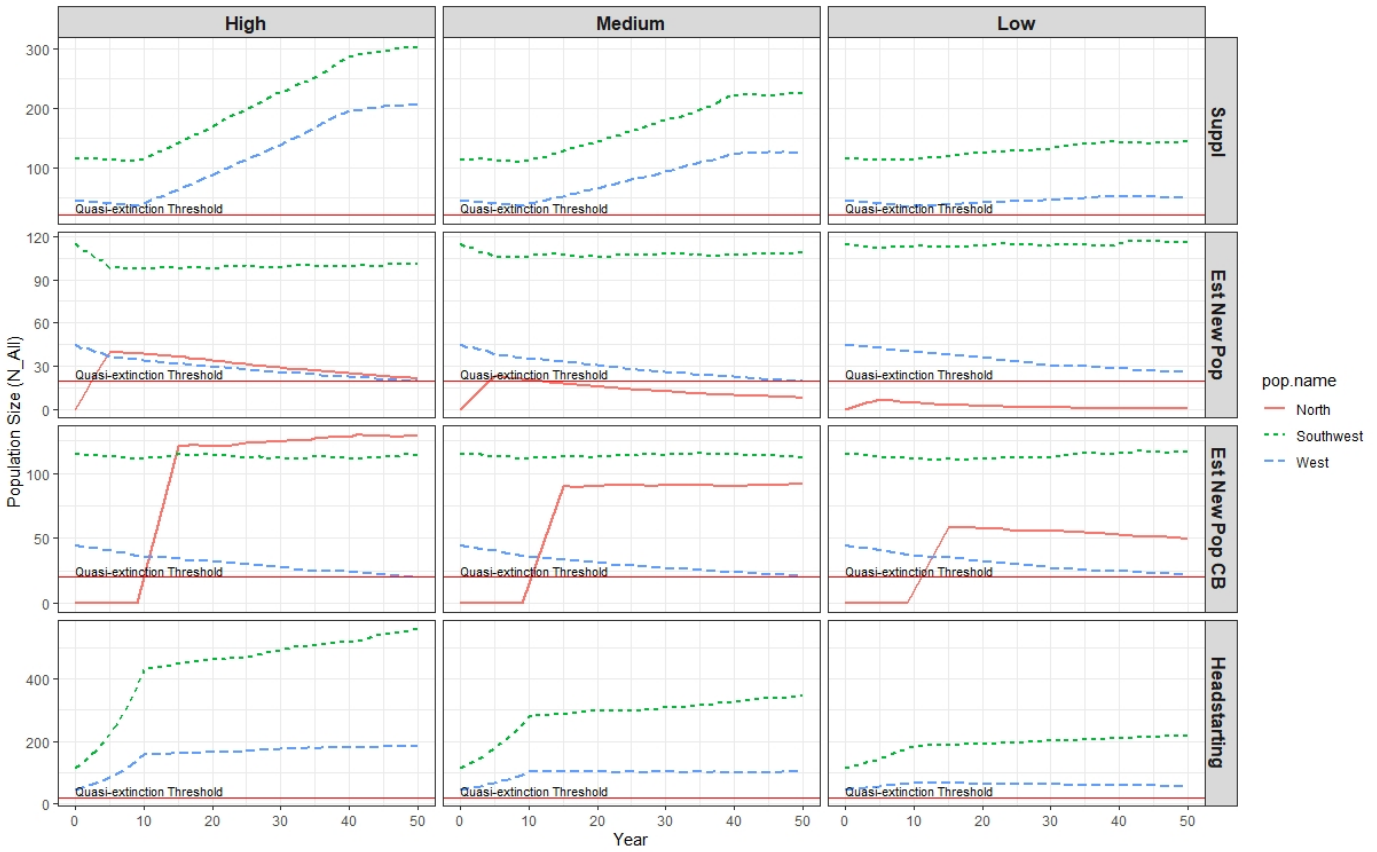

Figure 6.4: Predicted 50-year trends under different management scenarios showing Quasi-extinction threshold line in red intercepting the y-axis at 20 individuals. Suppl: Supplementation, Est New Pop: Establish New Population, CB: Captive Breeding.

6.5 Discussion

The outcome of the Grenada Dove baseline standard population model showed that the smaller population in the West has a declining population trend and a higher probability of quasi-extinction compared to the population in the Southwest. All threat scenarios increased the probability of quasi-extinction and showed a significant decrease in population growth rate for the populations they threaten. The most substantial threat to the Grenada Dove was the loss of habitat at Mt Hartman as proposed for commercial development. Population growth rates for supplementation and head-starting conservation management scenarios were all significantly higher and the population trajectories showed an increase for both populations. Head-starting appears to be the most effective conservation management strategy of the scenarios modelled for this species. When establishing a

new-population not all management scenarios produced a self-sustaining population, with some causing detriment to extant populations.

6.5.1 Standard Model

This PVA revealed a difference in population trajectories between the two different populations when modelling the standard baseline model with the smaller of the two having a decreasing population trend over the next 50 years. The population trend predictions for the both populations are congruent with population count data between 1987 and 2007 (Rusk, 2017) as the standard model was built to reflect current population trends. This difference was not unexpected given that smaller populations are more sensitive to stochastic processes (such as high levels of inbreeding and unstable age distributions) which are known to destabilise populations and can affect their viability (Lacy, 2000). The increased risk to this population highlights the importance of habitat protection in this area. Currently, a Dove Sanctuary established in 1996, which comprises of around 32 ha of the 113 ha of Perseverance Estate, is the only protected portion of habitat used by this population (Rusk, 2009, 2017). Despite being the smaller of the two, there is evidence to suggest this population has higher genetic diversity and unique genetic variation (Chapter 5), which further highlights the importance protecting this forest habitat and shows regardless of this higher genetic diversity the population in the West may still be vulnerable to stochastic processes.

6.5.2 Threat Scenarios

Modelling of threat scenarios indicate that the threat likely to have the largest negative impact is loss of dry forest habitat at the Southwest population. It is predicted that if the proposed area of Mt Hartman Estate is lost to commercial development then the Grenada Dove population in this area will become extinct within 5 years. This controversial development was first proposed in 2006 and to date, despite multiple proposals, has been prevented from going ahead (BirdLife International 2006; Rusk 2008, 2009; Rusk *et al.* 2010; Rosenberg 2018). Tropical dry forest is globally threatened and the Caribbean has already lost more than 60% of this forest type, despite the importance of this

ecosystem to local communities (Portillo-Quintero and Sánchez-Azofeifa 2009; Nelson *et al.* 2018). Insular tropical dry forest also has high levels of species endemism (Nelson *et al.*2018). Proposed loss of forest at Mt Hartman estate, home to one of Grenada's endemic species and only Critically Endangered bird (BirdLife International, 2022a; Blockstein, 1991; Rusk, 2009), will result in further fragmentation of Grenada Dove habitat. Habitat fragmentation poses a greater risk to small populations as it increases habitat edge effects which amplifies vulnerability to invasive predators, climate change and natural disaster increasing the likelihood of local extinction (Lees *et al.* 2022). This further underscores the need for environmentalists to be consulted when planning developments in order to prevent the loss of another species to anthropogenic habitat fragmentation (Brand and Thomas 2005; Dri *et al.* 2021; Lees *et al.* 2022).

Modelling of disease had a significantly negative effect on both populations, however, was simulated using data on general avian epidemics (Bruford *et al.* 1991). While disease is listed as a threat to the Grenada Dove, type and prevalence is unknown. Exotic diseases and pathogens have been known to have substantial effect on the survival of island birds due to their increased susceptibility (Wikelski *et al.* 2004). Examples of diseases threatening island species include *Trichomonas gallinae* and the Galápagos Dove *Zenaida galapagoensis*, avian malaria and the Mōhua *Mohoua ochrocephala* (Derraik *et al.* 2010) and *Mycobacterium avium* and the Hawaiian Goose *Branta sandvicensis* (Cooper and Path 1993; Wikelski *et al.* 2004). Therefore, screening for disease is required to identify potential pathogens/ diseases in the Grenada Dove population (Bolton *et al.* 2015) with implementation of specific disease control as appropriate (Thomas *et al.* 2007). Should data become available, modelling scenarios of specific diseases threatening the Grenada Dove would increase accuracy and provide information to predict and prevent outbreaks for future disease management strategies (O'Reilly *et al.* 2011).

While the model to assess the impact of a male-skewed sex ratio on this wild bird population shows an accelerated extinction risk in comparison to a 1:1 sex ratio, this scenario was modelled using the value reported for Critically Endangered birds (Donald, 2007), and in reality this may be less

extreme. Given the population trajectory since 1987 (although based on 1:1 sex ratio) has not declined into extinction over the past 35 years (Rivera-Milán *et al.* 2015; Rusk 2017), a heavily male-skewed sex ratio is unlikely. However, if there is a male-skewed sex ratio for this species, the outlook may be worse than currently thought as skewed ASRs elevates risk to small and isolated populations (Donald, 2007). Reasons for skewed ASRs are varied and include a correlation between sex ratio and population density (J. Gerlach & Maitre, 2001), a consequence of inbreeding (Postma *et al.* 2011) or a shortage of resources in which the males outcompete the females (Kilner, 1998). Molecular sexing results from Chapter 5 indicate a male skewed sex ratio of 3.6:1 in the samples used. However, given that samples were collected opportunistically, with no information available in regards to effects of age, sex, climate and breeding cycles on moult, inferences cannot confidently made about the extent of the sex ratio. The results of this PVA re-iterate the conservation and research recommendations made by Bolton *et al.* (2015) to determine the sex ratio for this species and hence more accurately assess the risk to Grenada Dove.

6.5.3 Management scenarios

When modelling conservation management strategies, the medium 10-year head-starting scenario produced viable populations in both the Southwest and West, with a significant increase in population growth rate. This management strategy has been successful for other bird species with a high risk of egg and nest predation for example, the Near Threatened Black-tailed Godwit *Limosa limosa* (Eaton & Holling, 2020), the Critically Endangered Mangrove Finch (Cunninghame *et al.* 2015) and the American Oystercatcher *Haematopus palliates* - a species of high conservation concern in south-eastern USA (Collins *et al.* 2016). However, this strategy may have limitations when applied to pigeons and doves due to practical issues in hand-rearing chicks such as replication of crop milk required to feed them (Ding et al., 2020; Swinnerton, 2001). Supplementation of birds in the wild using a 10-year captive breeding and 30-year release programme also significantly increased population growth rate, however, with lower final population trajectories than the head-starting management strategy. This suggests that juvenile survival may be a vulnerable life stage for the

Grenada Dove and increasing survival of this age group is important for effective conservation management. Notably, however, there is no available data on Grenada Dove juvenile survival so this was modelled using data from the closely related White-tipped Dove (Giese *et al.* 2018) assuming populations of this species, due to similarities in life-history traits (i.e. reproductive system), would have a similar response. By using information inferred from the *Leptotila* genus which is both a relatively data deficient group of birds (Peters et al., 2022) and also a group comprised largely of continental birds whose life-history traits are often known to differ from those of island species (Beauchamp, 2021; S. Clegg, 2010) means applying these values to the Grenada Dove was done with caution. Obtaining survival data along with quantified impacts of introduced predators on the Grenada Dove would be required prior to a head-starting programme (Bolton *et al.* 2015; Dolman *et al.* 2015; Collar 2020). While head-starting may be a novel and cost-effective conservation strategy, particularly to protect against introduced predators, it is imperative that it is conducted alongside *in-situ* predator control measures to ensure release of birds into the wild is effective (Dolman *et al.* 2015; Ross *et al.* 2021).

Only when using a hypothetical captive breeding programme did establishing a new population produce a viable population, however, this had a significant negative effect on extant populations used to establish the breeding programme. Establishing a new population without a captive breeding programme, on the other hand, failed to produce a viable population. The establishment of a new population is proposed in the species recovery plan for the Grenada Dove (Rusk *et al.* 2008) and has many benefits such as safeguarding against natural disasters such as hurricanes and maintenance of genetic diversity (Gregory *et al.* 2012; du Plessis *et al.* 2019) if implemented correctly. Establishing a new population using a captive breeding programme but with large scale supplementation of all populations over decades, may be a viable option for generating self-sustainable populations (Swinnerton 2001; Bowker-Wright *et al.* 2012; Gregory *et al.* 2012). This, however, was not modelled here as all scenarios were modelled independently due to the uncertainty of the synergistic effects of combining scenarios (Reed *et al.* 2003; Lacy 2019). Re-introduction of

birds needs to be carefully considered as complexities such as: ensuring there is suitable habitat to release birds, genetic management, generation of species specific rearing, establishment and breeding protocols, appropriate release techniques and post-release management, suitable funding and resource availability, along with any accompanying *in-situ* management – all of which needs to be taken into account prior to this type of management to help ensure its success (Black 1991; Swinnerton 2001; Dolman *et al.* 2015; Collar 2020).

PVAs are only as accurate as the data used to parameterize them (Reed *et al.* 2003; Lacy 2019). While it is possible to use imputed data and values from closely related species when there is no alternative – as has been the case here – in order for the population trajectories to be as realistic as possible the most accurate input values are required (Lacy, 2019). As such, realistic estimates of the probability of extinction rely on the quality and quantity of data available (Reed *et al.* 2003). For example, this study assumes a relatively slow breeding rate for the Grenada Dove, based on imputed data from Chapter 4 using life history information inferred from other members of the *Leptotila* genus (Peters et al., 2022), however, this is not typical of pigeons which in some cases are known to have long breeding seasons and produce multiple clutches per year (Gibbs et al., 2010) meaning the estimated two broods per year for the Grenada Dove may be an underestimate. This PVA highlights the vital need for information in regards to reproductive and mortality rates as well as data on the severity of catastrophes on reproduction and survival for this Critically Endangered species. As briefly mentioned above (6.5.3 Management scenarios), all scenarios were modelled individually due to the both the limited data available and the uncertainty of combining scenarios (Reed *et al.* 2003; Lacy 2019), and therefore may be lacking the complexities of reality, as such, it is reasonable to expect the outcome of management scenarios in particular will be changeable when combined with simultaneous negative impact of threat scenarios. Additionally, this model does not incorporate environmental stochasticity or genetics associated with allele transmission, however, this is not to say that these processes would not have an effect on population fluctuations or growth rates and when data is available should be incorporated into future models (Tenhumberg *et al.* 2004). This PVA

is not exhaustive and a number of other management techniques such as, cross-fostering (Richardson *et al*., 2004), supplementary feeding (Perry *et al.*, 2017) and disease screening and treatment (Swinnerton, 2001) may be effective for increasing the productivity of the Grenada Dove (Jones & Merton, 2012). Additionally, this PVA has only addressed biological factors associated with conservation management of the Grenada Dove, however, there are many non-biological factors - such as funding, resources, set up of breeding/rearing facilities, staffing considerations, project administration and stakeholder participation – which are necessary for the success of conservation programmes (Black, 1991; Swinnerton, 2001). It is recommended that future research should focus on trialling management techniques to determine which practises are possible with the resources and expertise available on Grenada.

6.6 Conclusion

This study has provided evidence that the population in the West has an accelerated risk of extinction, that loss of Mt Hartman is a significant threat to the long-term survival of this species and that a head-starting conservation strategy is potentially the most effective way of increasing population numbers. All threat scenarios significantly decreased population growth while head-starting and supplementation management scenarios significantly increased population growth rates. Establishment of a new population, however, failed to produce a viable population when also considering extant populations. In light of these findings, the proposed commercial development at Mt Hartman Estate should be prevented from going ahead as this will have catastrophic effects on the largest breeding population of this species and almost certainly cause the extinction of the Grenada Dove. Population supplementation, whether from a captive breeding or head-starting programme should be considered in the planning and development of future species recovery plans. Finally, this study highlights that data related to survival and mortality rates are urgently needed for accurate assessment of population viability of this Critically Endangered species.

CHAPTER 7 DISCUSSION

Photo by Howard Nelson

Chapter Seven – Discussion and Future Research

7.1 Genetic Techniques for Grenada Dove Conservation Overview

The Critically Endangered Grenada Dove is the focus of conservation efforts from the Grenada Dove Conservation Programme, the Forestry and National Parks department of Grenada along with local and international collaborators (BirdLife International, 2021b). As discussed throughout this thesis, evidence of drivers of the population decline of the Grenada Dove are lacking and more biological and ecological information would be beneficial to its conservation (Bolton *et al.* 2015). Factors forcing wild populations, such as this one, toward extinction are deterministic (habitat destruction and anthropogenic driven risks) and stochastic (environmental, demographic and genetic factors) (Albeshr, 2016). Knowledge of a species' genetic variation is important for understanding population declines, particularly in wild populations, and can help identify strategies for future conservation management (Charmantier *et al.* 2014; Frankham *et al.* 2019). This project aimed to provide detailed information of the genetic composition of the Grenada Dove at both a species and population level, as well as predict the long-term viability of this Critically Endangered species. This section will briefly summarise the findings from each chapter and discuss what each approach has to offer in the future management of Grenada Dove conservation.

Chapter 2 used a systemic review of the literature to identify trends present in conservation genetics of island birds and highlight areas which require further research.

- The number of studies increased over time, with molecular methods focused on sequencing of functional genes and molecular sexing used the least, and mtDNA and other nDNA markers types used most frequently. Invasive sampling was the most frequently used, and no evidence was obtained to show that sampling method was related to species conservation status.
- A geographical bias was identified showing that the Oceanic realm was the most studied geographical area and that regions of high conservation priority, such as the Caribbean and Southeast Asian islands, appear to be understudied.

- The majority of research was conducted on Passeriformes, with under-representation of threatened species and, for example, island specialist species such as Columbidae, of which the large majority of threatened species are island restricted.

This review of island bird genetic research revealed important trends which may be influencing the extent to which this information is actively applied to the conservation of island bird populations. The findings of this study show an increase in popularity of conservation genetics studies, a trend which can potentially be attributed to the greater accessibility of molecular techniques (Groombridge *et al.* 2018). This study also identifies geographic bias, with Oceania being the most studied geographical realm and areas of high biodiversity and endemism being under-represented (Belle *et al.* 2019). Least Concern species were the focus of the most studies. Notably not all of the studies reviewed made conservation recommendations, possibly due to the gap which exists between genetic research and conservation management (Haig *et al.* 2016; Britt *et al.* 2018). Future genetic studies should include research which focuses on under-represented areas, whilst prioritising threatened species in order to carry out genetic research which directly informs conservation. In island birds such as the Grenada Dove (a threatened species belonging to the largely under-studied Columbiformes family, and endemic to the Caribbean island of Grenada) outstanding knowledge of genetic variation could prove crucial for species recovery programmes. This has been demonstrated in a number of species with small, isolated populations, such as, the South Island Robin (Grueber *et al.* 2017), Swainson's Hawks *Buteo swainsoni* (Wenny *et al.* 2006), Seychelles Paradise Flycatcher *Terpsiphone corvina* (Bristol *et al.* 2013), Galápagos Short-eared Owl *Asio flammeus galapagoensis* (Schulwitz *et al.*2018) and Laysan Teal *Anas laysanensis* (Reynolds *et al.* 2015)*;* in all of the above genetic information was used to make conservation management recommendations. This demonstrates that genetic assessment of threatened bird species is an important conservation priority, but only if this information provides recommendations that can be used by conservation practitioners. This will consequently have direct species management implications, while also helping to bridge the gap between conservation genetic knowledge and conservation action (Taylor *et al.* 2017).

Chapter 3 developed molecular protocols and methods to increase the utility of non-invasively collected samples for conservation genetics of the Grenada Dove.

- DNA concentration was significantly increased and DNA yield was improved in DNA extracted from non-invasively collected plumulaceous feather samples when using an optimised method.
- DNA quality and amplification success was improved with the use of a primerless PCR technique when targeting mtDNA.
- The small amplicon strategy proved effective for DNA amplification of three overlapping regions of target DNA, which when combined, were used to sequence the 650 bp barcoding region of avian mtDNA for genetic analysis.

These methods were used to effectively obtain usable genetic material from moulted plumulaceous feather samples. As plumulaceous feather samples are considered non-optimal for genetic analysis they are often discounted for use in conservation genetic research (Presti *et al.* 2013; Vili *et al.* 2013). Along with sample type, the environmental conditions the samples were in can affect the quality of the samples (Hogan *et al.* 2008; Vili *et al.* 2013). Such conditions include hot and humid environments which often causes degradation to the DNA present in a sample (McNally *et al.* 1989; Sangali and Brandelli 2000; Hanson and Ballantyne 2005; Broquet *et al.* 2006). The methods outlined here were successfully applied to moulted-feathers and egg-shells that were non-invasively and opportunistically obtained, and which were collected in non-optimal conditions for samples intended for genetic analysis. Using these methods information regarding the phylogenetic placement (Chapter 4) and population genetics (Chapter 5) for the Critically Endangered Grenada Dove were obtained. The development of these methods is not only relevant to feather samples but all non-invasively collected samples, and therefore can be applied to a variety of species which are the focus of non-invasive methods. Examples include, the use of buccal swabs in the Near-Threatened White-crowned Pigeon (Cambrone *et al.* 2022), scales of teleost fish (Shuttleworth & Oosthuizen, 2022) and saliva deposited on the surface of a collection medium for Australian Brushtail Possums *Trichosurus vulpecula* (Emami-

Khoyi *et al.* 2021). In particular, the optimised DNA extraction protocol developed here was used to extract DNA from buccal swabs which were used to assess levels of inbreeding for the long-term genetic monitoring of reintroduced populations of Natterjack Toad *Epidalea calamita* (Phillips *et al.* 2020). While the use of non-invasive samples is well established (Andrews *et al.* 2018), to the best of my knowledge, this study was the first to demonstrate the use of these techniques to achieve successful extraction and amplification of informative DNA regions from degraded non-invasive feather samples for subsequent downstream conservation genetic applications.

Chapter 4 used phylogenetic methods to estimate the evolutionary history and phylogenetic placement of the Grenada Dove among other Neotropical columbids, and used phylogenetic comparative methods to predict life history traits.

- The first genetic data obtained for the Grenada Dove identified this species as a genetically distinct taxon with its phylogenetic placement on an individual branch, not shared by any of the other *Leptotila* species, and thus suggesting single lineage.
- The Grenada Dove shared a most recent common ancestor with its sister group containing *L. cassinii* and *L. plumbeiceps* with divergence approximately 2.53 mya, with a shared evolutionary history possibly with an upwards dispersal event by shared ancestors from South America to Central America, and the southern islands of the Lesser Antilles.
- Life history trait values predicted using phylogenetic imputation for the Grenada Dove were clutch size, the number of clutches per year, incubation time and hatching weight, with single imputation methods used to predict fledging age and longevity.

By understanding phylogenetic relationships within genus and family groups, a greater awareness of speciation and evolutionary history is gained, which can be fundamental to the conservation of threatened species (Johnson and Clayton 2000b; Johnson *et al.* 2001). Isolated island populations of Hook-billed Kite *Chondrohierax uncinatus* had lower levels of mtDNA genetic variability, as evidence

by phylogenetic analyses carried out by Johnson *et al.* (2007), who subsequently identified these populations as high conservation priorities for this species. Additionally, a re-classification of species status for the Cuban population to *Chondrohierax wilsonii* was also recommended as a result of this phylogenetic analysis, along with the need to assess life-history constraints in order to assist with the long-term conservation of Neotropical raptors (Johnson *et al.* 2007b). In an island species such as the Grenada Dove - which has a very limited range in comparison to species which were phylogenetically identified as closely related - increasing suitable habitat was recommended as a conservation priority by using closely related and Least Concern species as successful conservation models. Furthermore, evidence of monophyletic status supports the decision made to reclassify the Grenada Dove as a distinct species (Blockstein & Hardy, 1989), thus supporting its assigned conservation status of Critically Endangered (BirdLife International, 2021b). The use of predictive models, such as phylogenetic imputation of missing data, are particularly important for estimating outstanding information for rare and elusive species, species in remote and difficult to access locations, or where funding and resource constraints limit field surveys (Wood *et al.* 2018; Horswill *et al.* 2021). The largest vertebrate group of data-deficient species are amphibians, and as such González-del-Pliego *et al.* (2019) used geographic distribution data, phylogenetically imputed ecological traits, and an amphibian phylogeny to predict the threat-status for data-deficient amphibians. This highlights, not only which species and geographical locations that are at the highest risk of extinction, but also that often the most poorly known species are perhaps the most vulnerable. Life history traits essential to conservation management programmes - for example, longevity, age to maturity, clutch size, incubation and fledging time - were predicted for the Grenada Dove. However, using phylogeny rather than ecological determinates to predict life history traits has its limitations, particularly in island-dwelling species, and it has been documented that after accounting for phylogeny there can still be island effects for traits such as clutch size incubation time and growth traits (Clegg & Owens, 2002; Covas, 2012; Sandvig *et al*., 2022). Nevertheless, these predicted traits proved useful for further studies such as PVA (Chapter 6) in estimating a species' adaptability to environmental change as well

as predicting population growth rates over time (Mace *et al.* 2008; Pearson *et al.* 2014; Storchová and Hořák 2018; Cuervo and Møller 2019; Horswill *et al.* 2021).

Chapter 5 used genetic methods at a population level to assess the mitochondrial genetic diversity and population connectivity between two populations of the Grenada Dove.

- Examination of two mitochondrial markers has shown low mitochondrial genetic diversity in populations of the Grenada Dove.
- Genetically isolated populations of this species were identified with evidence that suggests past historical connectivity and subsequent isolation of these two populations.
- Molecular sexing revealed a male-sex bias in the Grenada Dove samples used.

These results highlight the importance of maintaining genetic diversity in small, threatened populations (Hudson *et al.* 2000; Fraser 2017). Analyses are indicative of a non-expanding population further supporting evidence from population estimation studies (Rivera-Milán *et al.* 2015; Rusk 2017) that populations of this species are not increasing. Genetic divergence between geographically sub-divided populations, as evidenced here, suggests that there is little to no gene flow between the populations, a problem which can increase the risk of extinction for small populations whose genetic diversity can erode as a result of inbreeding (Kahilainen *et al.* 2014). Information with regards to the genetic composition of populations can be used to make recommendations on how best to conserve populations and avoid inadvertent genetic consequences (Woodworth *et al.* 2002; Allendorf *et al.* 2007; Raisin *et al.* 2012). This approach has been used for the Purple-crowned Fairy-wren *Malurus coronatus,* whereby evaluation of observed genetic divergence was consistent with isolation by a natural barrier to gene flow; in this case the likely cause being widespread habitat degradation. In order to prevent further declines this study recommends conservation management for separate populations, along with habitat preservation to restore connectivity between isolated populations (Skroblin *et al.* 2012). Habitat protection, establishing new populations, and augmentation of gene

flow by translocation is recommended in order to increase genetic resilience and diversity of populations of the Grenada Dove, thus alleviating the extinction risk associated with low genetic diversity.

The sex ratio in Grenada Dove populations has been reported as parity (1:1), yet, although it is often stated that a male-biased sex ratio is suspected in this species (Bolton *et al.* 2015; Rivera-Milán *et al.* 2015; Rusk 2017) no studies previous to Chapter 5 have been conducted to investigate this. Molecular sexing allows reliable and rapid sex determination and is particularly useful for species which are sexually monomorphic and for samples of an unknown origin (Huynen *et al.* 2003; Patiño *et al.* 2013; Presti *et al.* 2013; Hatakeyama *et al.* 2020). A male-sex bias in Grenada Dove populations would mean that previous population calculations, which assume a 1:1 sex ratio, are over-estimated and therefore the population size of the Grenada Dove could be much smaller than predicted. In addition, male-sex bias in Critically Endangered wild bird populations are thought to increase a species' risk of extinction (Chapter 6; Donald 2007; Albeshr 2016).

Chapter 6 used population viability analysis to predict the effects of stochastic demographic events and conservation management approaches over a 50-year period for the Grenada Dove.

- The baseline standard population model showed a declining population trend for the smaller population in the West, with a higher probability of quasi-extinction compared to the population in the Southwest, which was relatively stable.
- Modelling threat scenarios revealed that all threat scenarios significantly decreased the population growth rate, with loss of habitat at Mt Hartman being the most substantial threat to the Grenada Dove.
- Modelling of conservation management strategies showed that supplementation and head-starting scenarios significantly increased population growth rates, with head-starting being the most effective management strategy.

This analysis highlights the importance of accurate estimation of extinction risk, along with informed selection of conservation management decisions to prevent further declines of wild populations (Reed *et al.* 2003; Olah *et al.* 2016; Lacy 2019). Deciding on appropriate conservation actions requires a good understanding of the causes of population decline, as well as biological and demographic data for accurate parameterisation (Olah *et al.* 2016; Ryan 2021). This approach has been used to predict the extinction risk for the wild Pink Pigeon population, by incorporating microsatellite data to assess the impact of genetic rescue. The results found that Pink Pigeons need both genetic rescue and continued population management, thus highlighting that a combination of genetic and demographic rescue are required to ensure the survival of this species (Ryan 2021; Jackson *et al.* 2022). Using original information generated by this study (Chapter 4 and Chapter 5), published data, expert knowledge of the Grenada Dove, along with published data on closely related species, the first PVA (to the best of my knowledge) was conducted for this Critically Endangered species. The threat scenario which appeared to have the largest impact on the long-term survival of the Grenada Dove was loss of habitat in the Southwest, providing evidence that the proposed commercial development at Mt Hartman Estate should be prevented, as this will almost certainly cause the extinction of the Grenada Dove. The head-starting conservation strategy was the most effective way of increasing population numbers, and as such is a management strategy that should be considered in the planning and development of future species recovery plans. Only the establishment of a new population scenario failed to produce a viable population when considering extant populations, however, this conservation strategy is already being recommended in the species recovery plan, stressing the importance of modelling techniques for identifying both effectual and ineffectual management strategies (Chaudhary & Oli, 2020). It would be greatly beneficial to have good-quality information on both survival and mortality rates, and environmental variables in order to improve the accuracy of such models and thus ensure effective conservation actions into the future (Reed *et al.* 2003; Tenhumberg *et al.* 2004; Lacy 2019).

This project has shown the importance of genetic information for conservation of Critically Endangered species, with both threatened species, and under-represented island regions (such as the Caribbean) being revealed as focus areas which would most benefit from genetic research (Chapter 2). The optimisation of molecular techniques has increased the utility of non-invasively collected samples, thereby reducing the need for invasive and potentially stressful sampling methods, something particularly useful for sensitive and Endangered species (Chapter 3). These techniques were applied to a little-known Critically Endangered Caribbean endemic, the Grenada Dove. Through the use of mitochondrial and nuclear DNA markers, the genetic status and phylogenetic placement of this species was identified and this data was subsequently used to predict missing life-history trait data (Chapter 4). Mitochondrial markers were used for haplotype analysis and assessment of genetic diversity at a population level, along with the use of sexing markers to determine the sex ratio of this species (Chapter 5). While making their own conservation recommendations in regards to habitat protection and restoration, as well as augmentation of gene flow, these genetic data were also used in a PVA (Chapter 6), allowing the identification of major extinction risks, along with effective conservation management strategies. In this regard, further research into the future trajectories of the Grenada Dove might benefit from the inclusion of specific genetic (e.g. inbreeding coefficients (Ryan, 2021)), environmental (e.g. carrying capacity (Kim *et al.* 2016)) and demographic (e.g. survival rates (Forys & Humphrey, 1999)) factors. Nevertheless, this project has provided initial information in regards to the genetic composition of the Grenada Dove, both at a species and population level, and has applied this novel material to the prediction of the long-term viability of this Critically Endangered species.

7.2 Sampling Methods for Conservation Genetics

This PhD demonstrated that non-invasively and opportunistically collected samples, which are often considered unsuitable for genetic analysis, can be used for downstream conservation genetic applications for wild populations, including phylogenetics (Chapter 4) and population genetics (Chapter 5). The Grenada Dove is particularly difficult to sample, with low encounter rates, cryptic

behaviour and sensitivity to environmental disturbance, and given its Critically Endangered conservation status, non-invasive sampling was the preferred approach as it caused minimal-to-no disruption to the species and was therefore considered more ethical (Segelbacher 2002; Bolton *et al.* 2015; Rivera-Milán *et al.* 2015; Rusk 2017). A variety of factors including resources, expertise and funding meant that organisations in Grenada were limited in the conservation management actions that could be carried out for the Grenada Dove, with genetic research neither viable nor possible. This international collaboration, therefore, allowed genetic research to be conducted and highlights the importance of international collaborations and partnerships with national and local conservation practitioners to aid on-the-ground conservation. However, this is only effective if the genetic research makes active and realistic conservation recommendations, that are effectively communicated to conservation partners thus circumventing the caveat of the 'conservation genetics gap' (Chapter 2; Hoban *et al.* 2013a; Taylor *et al.* 2017; Britt *et al.* 2018; Ryan 2021). The international nature of this collaboration meant that samples were reliant on collection by external partners, and was predominantly opportunistic, meaning that sample collection method, type and quality could not be controlled. Consequently, samples sent to the UK were often sub-optimal for genetic analysis and missing vital information such as collection site and date, with large numbers of samples grouped together in one sample bag; accordingly, the use of spatial and temporal information for classifying different individuals was particularly difficult. Underestimation of genetic diversity may arise from multiple replicates from the same individual (Bashalkhanov *et al.* 2009; Jackson *et al.* 2016; Schultz *et al.* 2021) so a number of samples were discounted in an attempt to control for repeat sampling of the same individual. Although non-invasively collected samples are considered more ethical (Baus *et al.* 2019) and may be the only option to sample a species (Zemanova, 2021), due to the uncertainty of the type, quality and condition of the samples (Segelbacher, 2002), adjustments to standard molecular protocols are often required to ensure their practical use in genetic research (Chapter 3).

Given the advances in non-invasive techniques and their usability, it is assumed that this type of sampling would be the preferred approach when taking into account ethical and welfare

considerations, particularly for threatened species (Taberlet and Luikart 1999; Russello *et al.* 2015; Baus *et al.* 2019). However, no relationship between conservation status and sampling method has been observed in sampling of island populations, and when considering wild populations of island bird species, non-invasive sampling methods appear to be used least frequently (Chapter 2). While it is thought that whole genome analysis will become routine in conservation biology (Postma *et al.* 2009; Groombridge *et al.* 2018; Britt *et al.* 2018; Mable 2019) such techniques are costly and often require large amounts of high-quality template DNA, something not achievable from non-invasively collected samples which typically yield low-quality DNA (Carroll *et al.* 2018). Genomic approaches, therefore, may not always be practical or cost effective (Monsen-Collar & Dolcemascolo, 2010), particularly in island populations that are under-represented (Chapter 2), a trait typical of developing areas (Martin *et al.* 2012; Meijaard *et al.* 2015) which may not have access to the funds and resources needed (Waldron *et al.* 2013). Consequently, while there appears to be a preference for non-invasive sampling as it is considered more ethical (Segelbacher 2002; Baus *et al.* 2019), there also appears to be a shift toward genomic techniques which, conversely, requires high-quality template DNA that is mainly provided through invasively collected samples (Carroll *et al.* 2018). Therefore, further developments of molecular techniques are needed in order improve the quality of template DNA and thus the biological value of non-optimal samples for genetic analysis; this is particularly important for wild populations of threatened, elusive and understudied species for which invasive sampling methods are not possible (Broquet *et al.* 2006; Hogan *et al.* 2008; Presti *et al.* 2013).

7.3 Genetics for Conservation

The incorporation of genetic information has fast become an integral part of conservation research and management (Weeks *et al.* 2011). Genetic assessment can improve insight into the genetic status of species of conservation concern (Pierson *et al.* 2021), levels of population connectivity (Ramírez *et al.* 2013), loss of genetic variation (Keller *et al* .2001), the presence inbreeding (Lawson *et al.* 2017), and can be used in the development captive-breeding programmes (Reddy *et al.* 2007). However, a gap between genetic research and its real-world application to conservation management has been

identified despite the widely acknowledged importance of genetic information (Chapter 2; Hoban *et al.* 2013a; Haig *et al.* 2016; Taylor *et al.* 2017; Britt *et al.* 2018). In order for genetic investigations to have maximum value to conservation, it is important that conservation genetic studies do not become a purely academic exercise (Britt *et al.* 2018) and that their findings are actively applied to practical conservation of the species they study. This has been demonstrated in a number of species with small, isolated, inbred populations whereby genetic rescue measures were effectually used to help save these species from extinction (Frankham *et al.* 2017) examples of this include the Greater Prairie Chicken *Tympanuchus cupido pinnatus* (Bouzat *et al.* 2009), Pink Pigeon (Jackson *et al.* 2022), South Island Robin (Heber *et al.* 2012) and Song Sparrow (Keller *et al.* 2001). Genetic rescue is a technique currently being used to prevent the extinction of the Endangered Eastern Bristlebird *Dasyornis brachypterus* whereby genetic assessment is underway and subsequent knowledge will be used to breed birds from different populations, with the aim of improving disease resistance, increasing genetic diversity and lifting fertility rates (Siossian 2021; Hogg *et al.* 2022). Such studies demonstrate the importance of genetic approaches and how they can positively influence conservation effects.

When considering the genetics of island birds, who are often at an increased risk from introduced disease (Wikelski *et al.* 2004), genetic isolation (Jouventin *et al.* 2006) and inbreeding depression (Swinnerton *et al.* 2004), it is important that missing information is identified and investigations that have the greatest benefit to their conservation are conducted. For some island species it may be that an increased effort in immunogenetic research, which appears to be under-studied in populations of island birds (Chapter 2) and may be beneficial for their conservation given the increased susceptibility of island species to disease (Ralls *et al.* 2020). In one such study, immunity toll-like receptor (TLR) genes were used to determine whether selection shaped genetic diversity in the Threatened Stewart Island Robin *Petroica australis rakiura.* Variation in TLR sequences is associated with defence against a wide variety of pathogens and resilience to infections (Gonzalez-Quevedo *et al.* 2015; Grueber *et al.* 2017), and when studying TLR diversity, Grueber *et al.* (2013) found that one particular genotype significantly improved the survival of individuals. They highlight

that genetic drift can be a determinant of genetic makeup associated with the innate immune system, and as such, isolated populations, which are more likely to experience genetic drift, should be managed to prevent loss of genetic diversity, as this may impact on the population's ability to respond to introduced infectious diseases (Grueber *et al.* 2013). Along with research on specific functional genes, having multiple or repeat studies of island bird populations was rarely observed (Chapter 2). Rather than investigating a single aspect of a species genetics, multiple studies could prove beneficial in providing information on wider aspects of their genetics, therefore having more conservation implications, and being useful for long-term genetic monitoring projects (Wan *et al.* 2004; Hu *et al.* 2011). In recent years there has been an increasing trend towards genomic research (Mable, 2019), however, this research is costly and requires specialist equipment and expertise which may be lacking in areas containing threatened island taxa. Consequently, focus and funding in such circumstances would be more useful if directed towards more realistic studies based on high priority conservation, and could therefore be directly incorporated into genetic management of the most deserving species (Hoban *et al.* 2013a, b; Di Marco *et al.* 2017; Davies *et al.* 2018).

Genetic research directed towards little-known island taxa, many of which are lacking any kind of genetic investigation, will also create a greater understanding of avian island genetics as a whole, and contribute to reducing taxonomic bias in conservation (Donaldson *et al.* 2016; Davies *et al.* 2018; dos Santos *et al.* 2020). Successful genetic management can cannot be effectively achieved without first obtaining fundamental knowledge of a species genetic status, population genetic profiles and demographic history (Liu *et al.* 2018; Siossian 2021). All genetic information was outstanding for populations of the Critically Endangered island endemic Grenada Dove. Using phylogenetic analysis, the distinct genetic status of the Grenada Dove was confirmed and light was shed on its evolutionary history. The newly identified sister taxa were used as a conservation model, assuming their Least Concern conservation status could be attributed to their extensive range, in comparison to the limited range of the Grenada Dove. Recommendations to restore dove habitat and increase its range were therefore identified as conservation priorities (Chapter 4; Peters *et al.* 2022). Assessing genetic

variation at a population level revealed low genetic diversity and isolated populations, providing evidence that the inclusion of augmentation of gene flow by translocation into Grenada Dove conservation strategies could be used to increase the population's genetic diversity and resilience (Chapter 5). Incorporating information resulting from this new genetic information, such as phylogenetically imputed life history trait data (Chapter 4), population genetic evidence of inbreeding and isolated populations, and sex ratio inferences from molecular sexing (Chapter 5), a PVA was conducted which identified threat scenarios posing the highest risk to Grenada Dove populations, along with predicting the most effective conservation management scenarios for this Critically Endangered species (Chapter 6). Outstanding genetic information pertaining to, for example, population structure, inbreeding depression, genetic relatedness, adaptive loci and levels of heterozygosity, could be obtained via the collection of microsatellites (Friesen *et al.* 2006; Hagen *et al.* 2011; Reynolds *et al.* 2015) and restriction site associated DNA (RAD) sequencing (Dierickx *et al.* 2015, 2019; Ryan 2021) data which, while potentially requiring higher sample quality (Carroll *et al.* 2018), would further aid the conservation management of the Grenada Dove.

7.4 Recommendations for Grenada Dove Conservation and Research

The fundamental aim of this PhD was to identify conservation recommendations using genetic data and assess the long-term viability of Grenada Dove populations. Habitat restoration and an increase in range (Chapter 4), along with extended habitat protection at Perseverance, which is a site containing unique genetic variation (Chapter 5), and Mt Hartman, a site at risk of commercial development (Chapter 6), are recommended. Upon identifying low genetic diversity and isolated populations (Chapter 5), a genetic rescue measure has been recommended via augmentation of gene flow by translocation of juveniles in order to increase genetic resilience and diversity, which if it is to have lasting beneficial effect, would require long-term genetic monitoring (Frankham *et al.* 2019). However, it is noted that the translocation of adults within islands is can be problematic and often using captive bred or head-started juveniles has a better success rate (Groombridge *et al.*, 2009). , For long-term genetic monitoring to be feasible, a multi-organisation approach may be needed due to the

lack of facilities required to carry out genetic techniques within national organisations currently working to conserve the Grenada Dove (Wilson *et al.* 2016; Wheeler *et al.* 2021; Hogg *et al.* 2022). Careful genetic management would also be required for establishing new populations, something which is recommended for forest habitat in the Northern most region of the island. This would require translocation of juvenile birds and would need to be supported post release with supplemental food and predator control at the newly established population to ensure survival (Swinnerton, 2001). While a new population here would be valuable for safeguarding against natural disasters such as hurricanes, and maintaining genetic diversity (Gregory *et al.* 2012; du Plessis *et al.* 2019), when modelled, the only viable option for establishing a new population was with population supplementation from a captive breeding programme (Chapter 6). As such the drastic population increase required is not possible with current wild population trajectories. Head-starting has been identified as the most effective conservation strategy, showing the greatest increase in population numbers in a fairly short amount of time. This strategy, however, would first need to be supported by specific data on Grenada Dove nest success and juvenile survival, along with quantified impacts of introduced predators (Chapter 6). While strategies such as head-starting and captive breeding are effective ways to produce young with which to supplement existing populations such actions need carefully considered and preferably consult experiences personal, for example, by twinning with similar projects (Collar, 2020; Dolman *et al.*, 2015). This technique has been successfully used for a number of island bird populations including Kiwi species *Apteryx spp*. (Colbourne *et al.* 2005; Jones and Merton 2012). Still, release of birds will only be successful if carried out alongside *in-situ* management thus ensuring suitable release conditions (Dolman *et al.* 2015; Collar 2020). All conservation management plans, however, may be futile as it has been predicted that loss of significant habitat to proposed commercial development could directly cause the extinction of the Grenada Dove (Chapter 6) and must be prohibited from going ahead if this species is to survive. Now may be the time for collaboration of national and international organisations to call for its prevention.

When deciding on appropriate conservation and action plans for the Grenada Dove it is important to look at successes and failures from conservation of other threatened island bird species (Copsey *et al.* 2018). One, predominately Caribbean, Endangered bird species which has benefited from intensive study and management action, is the Black-capped Petrel *Pterodroma hasitata* (Birds Caribbean, 2022), a poorly-understood and secretive species which was the focus of a conservation and action plan outlined in 2012 (Goetz *et al.* 2012). This prompted 10-years of monitoring and research including nest monitoring, radar surveys, habitat modelling and satellite tracking, allowing the identification of more than a hundred active burrows, an increased understanding of petrel movement at sea and improved knowledge of the natural history of this species (Wheeler *et al.* 2021). Genetic research included examination of population genetic structure of Black-capped Petrel and revealed genetic difference between dark and light morphs suggesting reproductively isolated populations (Manly *et al.* 2013). All of this newly gained information has contributed to an increased knowledge of this Endangered species and a more informed conservation action plan, which includes reducing predator pressure, exploring restoration methods and continued monitoring, in order to aid in securing the future of the Black-capped Petrel (Wheeler *et al.* 2021; Birds Caribbean 2022). It is hoped that the data presented in this thesis, and the subsequent discussions and recommendations, will contribute to the development of updated conservation and action guidelines for the Grenada Dove, and will be incorporated, both in theory and in practise, to recovery programmes contributing to the future survival of this species.

Data and information on fundamental vital rates including nest success, survival, mortality and sex ratio (Bolton *et al.* 2015), along with further genetic research, such as information on loci under selection and thereby understanding this species adaptive potential (Ryan, 2021), would all contribute to effective conservation management of the Grenada Dove. Obtaining information on survival rates could be achieved through a ringing programme which could provide information in regards to population turnover, longevity, movements and population numbers (Bolton et al., 2015; Hernández et al., 2018). By establishing a nest monitoring scheme, which are often facilitated through

the use of camera trapping techniques, information pertaining to the breeding biology and productivity could be obtained through documentation of nest success and causes of nesting failure (Bolton *et al.*, 2015; Gamero *et al.*, 2014; Militão *et al.*, 2017). Information in regards to the feeding ecology of the Grenada Dove could be observed by direct observations or through the use of non-invasively collected faecal samples and DNA metabarcoding techniques (Ruppert *et al.*, 2019). Additionally, by obtaining historical samples from museums, the current ecology and distribution could then be placed in the context of historical change as such specimens may provide further information about life history and collection locality (Schindel *et al.*, 2011; Spurgin *et al.*, 2014).

Predators control projects to reduce or eliminate exotic mammalian predators are important and often effective conservation management strategies for many Endangered bird species (Barun *et al.*, 2011; van Rees & Reed, 2018). To be effectual predator control techniques need to be rigorous and professionally conducted (Barun *et al.*, 2011). As little is known about the impacts of exotic mammals on the Grenada Dove empirical data on the type, abundance and range of introduced predators would be useful for quantifying the predator effects and thus be used to inform a more intensive and systematic predator control programme (Rusk *et al.* 2008; Bolton *et al.* 2015; Rivera-Milán *et al.* 2015). This can be obtained by examining predators caught in the current predator control programme and taking morphometric measures and noting life stage and tooth wear as indicators of age, as well as recording sex, weight, condition and stomach contents in order to determine the feeding ecology of these species and detect whether they predate on birds and eggs of the Grenada Dove (Meckstroth *et al*., 2007; Walsh *et al.*, 2012).

As many pigeon and dove species are susceptible to *Trichomonosis* (Bolton *et al.*, 2015; Nancy Bunbury *et al.*, 2008), evidence of the presence and abundance of this among Columbidae species on Grenada would be informative for conservation efforts. In order to achieve this, a disease screening programme could be established whereby any birds being caught for ringing, nest monitoring or other such projects would be screened and blood samples taken to screen for blood parasites and for further genetic research (Bolton *et al.*, 2015; Swinnerton, 2001).

Evaluating carrying capacity of available habitat would be useful to guide restoration actions and in increasing available range to accommodate population expansion (Goss-Custard *et al.* 2002; Ruarus *et al.* 2011; Rivera-Milán *et al.* 2015). Given that the Grenada Dove lives in secondary forest, appears to favour areas that have regenerated after hurricanes and spends a lot of time foraging on the on the forest floor, studies to determine microhabitat characteristics and whether the dove is responding to successional stage are required to better understand the relationship between the dove and the dry forest ecosystem (Lugo, 2005; Rusk *et al.*, 2008). Limited resources, including lack of water, are listed as a risk factors for this species, as such, installation of additional wells and leaky hose pipe as well as further investigation into the limiting factors of this habitat is recommended (BirdLife International, 2021b; Bolton *et al.*, 2015).

It is important to note that, in addition to lack of biological knowledge, a variety of significant aspects such as funding, skills and expertise, resources and facilities, are often constraints to many conservation programmes (Black, 1991; Swinnerton, 2001) and is likewise the case for the Grenada Dove (Rosenberg and Korsmo 2001; Lugo 2005; Rusk *et al.* 2008). A multi-organisational approach to this species conservation is imperative, with collaborations helping to lessen some of these limiting factors (Devenish-Nelson *et al.* 2019; Wheeler *et al.* 2021; Hogg *et al.* 2022). This could be achieved by effective collaboration, information flow and training between the Grenada Dove Conversation programme and other similar conservation efforts such as the Pink Pigeon project (Albeshr, 2016; Ryan, 2021; Swinnerton, 2001). Ultimately, a combination of genetic monitoring and demographic rescue management techniques (Liu *et al.* 2018; Jackson *et al.* 2022) is an approach that should be undertaken to assure the future of the Critically Endangered Grenada Dove.

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Appendix I: Systematic Review Inclusion and Exclusion Criteria

Table **1: Database Advanced Search Criteria**

Database	**Time Frame**	**Language**	**Document Type**
Web of Science (WoS) Clarivate (2020)	All years (1970-2020)	English	Article, Other, Data study, Early access, Case report, Unspecified
Bielefeld Academic Search Engine (BASE) Bielefeld University Library (2020)	All years (1970-2020)	English	Article contribution; Report; Manuscript; Master's thesis, doctoral and postdoctoral thesis; Unknown.

Table **2:** '*Gold Standard'* hit results for checking search strategy precision and recall as outlined in Grames *et al.* 2019 performed in RStudio using the litsearchr package (Grames *et al.*2019; R Core Team 2021)**.**

***'Gold Standard'* Article**	**Article found: Best match**	**Similarity**
[1,] "Population Genetics of the Galápagos Hawk (Buteo Galapagoensis): Genetic Monomorphism Within Isolated Populations"	[1,] "Population Genetics of the Galápagos Hawk (Buteo Galapagoensis): Genetic Monomorphism Within Isolated Populations"	[1,] "1"
[2,] "Signature of a pre-human population decline in the critically endangered reunion island endemic forest bird coracina newtoni"	[2,] "Signature of a Pre-Human Population Decline in the Critically Endangered Reunion Island Endemic Forest Bird Coracina newtoni"	[2,] "0.903225806451613"
[3,] "Geographic variation and genetic structure in the Bahama Oriole (Icterus northropi), a critically endangered synanthropic species"	[3,] "Geographic variation and genetic structure in the Bahama Oriole (Icterus northropi), a critically endangered synanthropic species"	[3,] "1"
[4,] "The origin of the world's smallest flightless bird, the inaccessible island rail atlantisia rogersi (aves: rallidae)"	4,] "The origin of the world's smallest flightless bird, the Inaccessible Island Rail Atlantisia rogersi (Aves: Rallidae)"	[4,] "0.948275862068966"
[5,] "Genetic diversity and extent of gene flow in the endangered Japanese population of Hodgson's hawk-eagle, Spizaetus nipalensis"	[5,] "Genetic diversity and extent of gene flow in the endangered Japanese population of Hodgson's hawk-eagle, Spizaetus nipalensis"	[5,] "1"
[6,] "Using ancient dna to quantify losses of genetic and species diversity in seabirds: a case study of pterodroma petrels from a pacific island"	[6,] "Using ancient DNA to quantify losses of genetic and species diversity in seabirds: a case study of Pterodroma petrels from a Pacific island"	[6,] "0.964028776978417"
[7,] "The complete mitochondrial genome of norfolk robin (petroica multicolor: petroicidae), an endemic endangered species in norfolk island"	[7,] "The complete mitochondrial genome of Norfolk Robin (Petroica multicolor: Petroicidae), an endemic endangered species in Norfolk Island"	[7,] "0.955223880597015"
[8,] "MATERNAL GENETIC STRUCTURE REVEALS AN INCIPIENT DIFFERENTIATION IN THE CANARY ISLANDS CHIFFCHAFF PHYLLOSCOPUS CANARIENSIS"	[8,] "MATERNAL GENETIC STRUCTURE REVEALS AN INCIPIENT DIFFERENTIATION IN THE CANARY ISLANDS CHIFFCHAFF PHYLLOSCOPUS CANARIENSIS"	[8,] "1"
[9,] "Interisland genetic structure of two endangered Hawaiian waterbirds: The Hawaiian Coot and Hawaiian Gallinule"	[9,] "Interisland genetic structure of two endangered Hawaiian waterbirds: The Hawaiian Coot and Hawaiian Gallinule"	[9,] "1"
[10,] "Phylogeography of three endemic birds of Maratua Island, a potential archive of Bornean biogeography"	[10,] "Phylogeography of three endemic birds of Maratua Island, a potential archive of Bornean biogeography"	[10,] "1"

Table **3: Study Inclusion and Exclusion criteria**

Category	Inclusion	Exclusions
Geographical location		
Islands	• Studies of bird populations on islands (land not considered continental mainland) • Studies where comparison is made between island populations and continental populations • Studies of island populations of island breeding birds	• Studies of bird populations on land considered continental mainland only • Studies of widespread species where no direct comparison was made between island population and continental populations
Taxa		
Taxonomic Information	• Studies investigating island populations of extant species	• Studies of extinct species • Studies of domestic and commercial breeds • Multi Class studies
Methodology		
Molecular Methods	• Studies that collected primary avian molecular data • Studies that collected primary avian molecular data and included open source data/ data from another study • Studies that used avian molecular sexing	• Studies that did not collect primary avian molecular data • Studies that used non-molecular methods to obtain primary data e.g. genetic algorithms, pedigrees, simulations • Studies using molecular methods for avian dietary analysis • Studies investigating avian virus and disease

Table **4: Review Categorisation Criteria**

Category	**Characterisation**	
Geographical Location		
Island	Name of Island(s)/ countries which are the focus of the study.	
Realm BirdLife International (2021)	Geographical realm(s) as specified by the BirdLife DataZone species fact sheet including: Afrotropical, Antarctic, Indomalayan, Nearctic, Neotropical, Oceanic, Palearctic.	
Region	Geographic region of focal islands	
Taxonomic Information		
Classification	Scientific classifications: Order, Family, Genus, Species were recorded Studies investigating greater than three of Order, Family, Genus or Species were recorded as MULTI species studies	
Species	Sub-species were not recorded and were instead recorded as their full species status Species used purely as out-groups were not recorded	
Conservation		
Conservation Status BirdLife International (2021)	Conservation status was recorded as current species conservation status according to BirdLife Data Zone species fact sheet Including: Extinct in the Wild (EW), Critically Endangered (EN), Endangered (EN), Vulnerable (VU), Near Threatened (NT) and Least Concern (LC)	
Category	**Yes**	**No**
Geographical location		
Mainland Vs Island	Studies conducted where direct comparison is made between island and continental populations	Studies conducted on island populations only Studies conducted on island populations of island breeding birds only
Island Restricted BirdLife International, (2021) The Cornell Lab of Ornithology (2021)	Species range, assessed using Birds of The World and BirdLife Data Zone, is restricted to islands	Species range, assessed using Birds of The World and BirdLife Data Zone, included continental range
Island Breeding BirdLife International (2021) The Cornell Lab of Ornithology (2021)	Island breeding species, as indicated by Birds of The World	Continental breeding species, as indicated by Birds of The World Terrestrial island restricted species, assessed using Birds of The World and Bird, Life Data Zone
Endemic BirdLife International (2021)	Species were considered endemic when specified as country endemic according to BirdLife Data Zone species fact sheet	Species not considered endemic when specified as country endemic according to BirdLife Data Zone species fact sheet Regional and continental endemics
Conservation		
Management recommendations	Studies which make active conservation management recommendations for the focal study populations/ species including defining sub-populations as Evolutionarily Significant Units (ESUs) Studies with a distinct conservation implications, recommendations and/or management section included in the discussion	Studies which do not active conservation management recommendations Studies with a conservation focus but do not make conservation management/ recommendations for the focal study populations

Taxonomic recommendations	Studies which recommend taxonomic revisions of study species	Studies which do not make taxonomic revision recommendations
Methodology		
Molecular marker/ data collected Groombridge *et al.*(2018)	Studies which collect data on the following molecular markers/ regions, grouped as follows: **mtDNA** (e.g. mitochondrial DNA markers, mitochondrial genome) **nDNA** (e.g. nuclear DNA markers, Microsatellites, Double Digest Restriction Associated DNA, Single-nucleotide polymorphisms (SNP's), Randomly amplified polymorphic DNA markers (RAPDs), Short tandem repeats (STR's), whole genome sequencing, etc.) **Functional Genes** (e.g. Major histocompatibility complex, Toll-like receptor, etc.) **Sexing** (Molecular sexing markers)	Studies which do not collect data on the following molecular markers/ regions: **mtDNA** (e.g. mitochondrial DNA markers, mitochondrial genome) **nDNA** (e.g. nuclear DNA markers, Microsatellites, Double Digest Restriction Associated DNA, Single-nucleotide polymorphisms (SNP's), Randomly amplified polymorphic DNA markers (RAPDs), Short tandem repeats (STR's), whole genome sequencing, etc.) **Functional Genes** (e.g. Major histocompatibility complex, Toll-like receptor, etc.) **Sexing** (Molecular sexing markers)
Sample Type		
Invasive	Samples collected using invasive methods e.g. blood and tissue samples	Sample collected using non-invasive methods e.g. feather samples, museum samples, by-catch samples
Non-invasive	Sample collected using non-invasive methods e.g. feather samples, museum samples, by-catch samples	Samples collected using invasive methods e.g. blood and tissue samples
Wild/ Contemporary	Samples collected from wild and/or considered contemporary	Samples not collected from wild and/or considered contemporary
Museum/ Ancient	Samples collected from museum specimens and or considered ancient	Samples not collected from museums specimen and or considered ancient
Captive	Samples collected from captive populations	Samples not collected from captive populations

Appendix II: Primer3 output example for designed primers

Primer3 Output

```
PRIMER PICKING RESULTS FOR

Template masking not selected
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm      gc%  any_th  3' th hairpin seq
LEFT PRIMER        174   21   58.32   52.38    0.00   0.00    0.00 ctgcctactaacccagatcct
RIGHT PRIMER       376   20   58.78   55.00    0.00   0.00    0.00 aggagccgtagtagagtcct
SEQUENCE SIZE: 1260
INCLUDED REGION SIZE: 250

PRODUCT SIZE: 203, PAIR ANY_TH COMPL: 5.69, PAIR 3'_TH COMPL: 5.69

    1 cctttgcactgtccatccttattattatcctatcactccacagacctaaaaccaatggcc
      ............................................................

   61 cccaacctacgaaaacatcaccctctactaaaaataattaacaactccctaatcgaccta
      ............................................................

  121 ccaacccccttcaaacatctctgcctgatgaaactttgggtccctattaggcatctgccta
      ...........................                           >>>>>>>

  181 ctaacccagatcctaacaggcctactactagccgcacactataccgccgacaccactcta
      >>>>>>>>>>>>>

  241 gccttctcgtccgtagcccatacatgccggaatgtacaacatggctgactaattcgtaac


  301 ctccacgcaaacggagcctccttcttcttcatttgcatctacttacacatcggacgagga
                                                              <<<<

  361 ctctactacggctcctacctgtataaagaaacctgaaacacaggagtcatcctcctacta
      <<<<<<<<<<<<<<<                       ......................

  421 accctaatagcaactgccttcgtggggtacgtcctaccctgaggacaaatatcattctga
      ............................................................

  481 ggcgccacagtcatcactaacctcttttcagctatcccttacattggccaaaccctagtc
      ............................................................

  541 gaatgagcctgaggcggattttccgtagataaccccacattaacacgatttttcacctta
      ............................................................

  601 cacttcctcctcccatttataatcgcaggtctcaccatcatccacctcaccttcttacat
      ............................................................

  661 gaatccggctcaaacaacccactaggtatctcctccaactgcgacaaaatcccattccac
      ............................................................

  721 ccctacttctccctaaaagacatcctaggcttcacacttatattcctccccctaacaacc
      ............................................................

  781 ttggccctcttctccccaacctcttaggagacccagaaaacttcacacccgcaaaccca
      ............................................................

  841 ctagtcacacctccccatatcaagccagaatgatacttcctattcgcatatgccatcctc
      ............................................................

  901 cgctccatccccaacaaactaggaggtgtactagccctagccgcctccgtactaatcctc
      ............................................................

  961 ttcctcacccccctactccacaaatctaaacagcgcacaataaccttccgccccctctcc
      ............................................................

 1021 caaatcctgttctgaaccctagtcgccaacctcctcatcctaacatgagtaggcagtcag
      ............................................................

 1081 cccgtagaacacccattcatcattatcggccaactagcctccctcacctactttaccatt
      ............................................................
```

```
  1141 atccttatcctcttccccaccatcggagccctagaaaacaaattacttaactactaaact
       ............................................................

  1201 ctaatagtttatataaaacatcggtcttgtaaaccgaagactgaaggctatacccttct
       ............................................................

KEYS (in order of precedence):
...... vector sequence
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS
                    start  len      tm      gc%  any_th  3'_th hairpin seq

 1 LEFT PRIMER        176   23   59.03   47.83    0.00   0.00    0.00 gcctactaacccagatcctaaca
   RIGHT PRIMER       380   21   58.87   52.38    0.00   0.00    0.00 aggtaggagccgtagtagagt
   PRODUCT SIZE: 205, PAIR ANY_TH COMPL: 17.53, PAIR 3'_TH COMPL: 5.45

Statistics
         con   too    in    in   not          no    tm    tm   high  high  high        high
         sid  many   tar  excl    ok   bad    GC   too   too any_th 3'_th hair-  poly   end
        ered    Ns   get   reg   reg   GC% clamp   low  high  compl compl   pin     X  stab    ok
Left     306     0     0     0     0     0     0   157    22      0     0    96     0     0    31
Right    306     0     0     0     0     4     0   175    15      0     0     0     0     0   112
Pair Stats:
considered 2898, unacceptable product size 2895, primer in pair overlaps a primer in a better pair 897, ok 3
libprimer3 release 2.4.0


(primer3_results.cgi release 4.1.0)
```

Appendix III: published version of Chapter 3

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METHODS AND RESOURCES ARTICLE

A novel method to optimise the utility of underused moulted plumulaceous feather samples for genetic analysis in bird conservation

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Abstract

Non-invasive sampling methods are increasingly being used in conservation research as they reduce or eliminate the stress and disturbance resulting from invasive sampling of blood or tissue. Here we present a protocol optimised for obtaining usable genetic material from moulted plumulaceous feather samples. The combination of simple alterations to a 'user-developed' method, comprised of increased incubation time and modification of temperature and volume of DNA elution buffer, are outlined to increase DNA yield and significantly increase DNA concentration ($W = 81$, $p < 0.01$, Cohens's $d = 0.89$). We also demonstrate that the use of a primerless polymerase chain reaction (PCR) technique increases DNA quality and amplification success when used prior to PCR reactions targeting avian mitochondrial DNA (mtDNA). A small amplicon strategy proved effective for mtDNA amplification using PCR, targeting three overlapping 314–359 bp regions of the cytochrome oxidase I barcoding region which, when combined, aligned with target-species reference sequences. We provide evidence that samples collected non-invasively in the field and kept in non-optimal conditions for DNA extraction can be used effectively to sequence a 650 bp region of mtDNA for genetic analysis.

Keywords Non-invasive sampling · Feather samples · Polymerase chain reaction (PCR) · Primerless PCR · mtDNA · Small amplicon strategy

Introduction

Working with cryptic, rare or elusive species can make obtaining invasive samples such as tissue or blood logistically difficult (Mills et al. 2000; Horváth et al. 2005). Moreover, for endangered and sensitive species, it can be difficult to obtain permits for more intrusive sampling methods, which in some cases are considered unethical (Segelbacher 2002). In these cases, biological samples such as feathers, hair, buccal cells, faecal matter and shed skin can be collected in the field with minimal disturbance to the study species (Mills et al. 2000; Bohmann et al. 2014). However, some types of non-invasive sample collection such as buccal swabbing and hair plucking still require trapping and handling of the animal (Broquet et al. 2006; Dai et al. 2015), and these methods have the potential to cause stress and affect the behaviour of an individual, even if such effects are short term (Broquet et al. 2006; Rudnick et al. 2009; Dai et al. 2015). Highly non-invasive sample collection of material such as moulted feathers, shed skin, faecal samples or environmental DNA (eDNA), which can be collected opportunistically in the field, can eliminate the need to interact with the study species (Bayard De Volo et al. 2008; Bohmann et al. 2014). This is advantageous, particularly for research on sensitive species and ecosystems, as it minimises the level of disturbance to the wildlife and prioritises the welfare of individuals being investigated (Dai et al. 2015).

Despite the advantages of using highly non-invasive sampling methods, it is often difficult to identify the biological material collected to species-level without genetic analysis

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(Mills et al. 2000; Rudnick et al. 2009). This is a particular problem for samples such as feathers, hair and faecal matter that can often come from a variety of species sharing the same habitat with similar somatic features (Waits and Paetkau 2005; Coghlan et al. 2012; Ahlers et al. 2017). Sample type also has an impact upon the likelihood of successful DNA extraction, for example, large primary, secondary and tail feathers are preferable for obtaining usable genetic material compared to smaller plumulaceous feathers (Dove 2000; Vili et al. 2013). Larger feathers are embedded deeper in the body of the bird and so are more likely to retain DNA containing biological material such as epithelial cells (Seki 2006; Gebhardt and Waits 2008). Primary feathers in particular can contain the umbilicus blood clot in the shaft of the quill which is a by-product of feather development and, if present, provides a plentiful source of DNA (Segelbacher 2002; Vili et al. 2013). However, opportunistic sample collection methods often remove the ability to select for sample type (Broquet et al. 2006). Furthermore, there is often no indication of how long the sample has been in the field and thus exposed to a variety of environmental conditions before collection (Hogan et al. 2008; Vili et al. 2013). Hot and humid environments provide non-optimal conditions for biological samples intended for genetic investigation (McNally et al. 1989; Hanson and Ballantyne 2005) as this can lead to a higher prevalence of decomposing microorganisms such as fungi, mould spores and keratin-degrading microorganisms, which can damage the DNA (Sangali and Brandelli 2000; Vili et al. 2013). The increased likelihood of degraded DNA in such samples reduces and often prevents the selection of non-invasively collected samples for use in genetic analyses (Vili et al. 2013). Therefore, an improved method is needed to increase the biological value of low quality samples which have been kept in non-optimal conditions; particularly for endangered or elusive species for which invasive sampling methods are not possible (Broquet et al. 2006; Hogan et al. 2008; Presti et al. 2013).

In this study we focused on the utility of non-invasively collected feather samples of the critically endangered Grenada dove (*Leptotila wellsi*). We provide a three-step process to allow successful extraction and amplification of mitochondrial DNA (mtDNA) from plumulaceous feather samples. Firstly, we describe improvements to a user-developed protocol for DNA extraction that increases DNA yield, followed by primerless PCR to improve quality, along with a small amplicon strategy that enabled effective mtDNA amplification using PCR, targeting three overlapping regions of the cytochrome oxidase I (COI) barcoding region.

Methods and results

Study species and sample collection

The Grenada dove is a critically endangered columbid, endemic to the island of Grenada (Rosenberg and Korsmo 2001; BirdLife International 2019) with a population size of 160 ± 30 individuals (Rivera-Milán et al. 2015). Habitat loss and degradation means that this species exists in small fragmented populations (Rosenberg and Korsmo 2001; Rusk 2008; Rivera-Milán et al. 2015). It is threatened by predation from a number of mammalian species as well as habitat destruction from natural damaging events such as hurricanes (Bolton et al. 2016). Low encounter rates and cryptic behaviour make surveying this species particularly difficult, requiring intensive monitoring that may cause disturbance (Rivera-Milán et al. 2015; Bolton et al. 2016; Rusk 2017). Therefore, non-invasive sampling methods are required to obtain samples for genetic analysis with minimal disruption to this endangered species.

Feather samples used for this study were obtained non-invasively, as moulted feathers, and collected opportunistically from known occupied dove territory: Mount Hartman estate and Perseverance, Grenada (Rusk 2008, 2017). Habitat consists of both dry and mixed broadleaf evergreen-deciduous second-growth forests (Rusk 2017). This tropical dry forest habitat has a minimum temperature of 22 °C and temperatures that can reach up to 32 °C, with a maximum rain fall of 259 mm in the rainy season and a minimum of 67 mm in the dry season (Meteostat 2018; Nelson et al. 2018). Due to the opportunistic nature of the sample collection, the feathers used in this study spent an unknown amount of time in the litter bed of this hot and humid environment before collection. Samples were stored in sample bags at 4 °C until transportation by airmail to the UK in June 2018. On arrival in the UK, the samples were cleaned with 70% ethanol and stored at − 20 °C. The sample set (n = 160) used in this study was comprised of 152 plumulaceous

Fig. 1 Examples of the plumulaceous feathers used in this investigation

(Fig. 1), three secondary and three primary feathers, as well as two egg shells recovered from the forest floor.

DNA extraction

The commercially available QIAGEN DNeasy® Blood and Tissue kit was used to conduct DNA extraction. Extractions were carried out as per the 'User-Developed Protocol: Purification of total DNA from nails, hair, or feathers using the DNeasy® Blood & Tissue Kit' (QIAGEN Inc., Crawley) with the following alterations to incubation time, temperature and volume of DNA elution buffer. Feather samples were cut into 1 cm pieces directly into a sterile 1.5 ml microcentrifuge tube containing the lysis buffers using sterile scissors to increase surface area (Presti et al. 2013). The incubation step was increased to 48 hours in order to achieve complete sample lysis on samples that are particularly difficult to digest (Bush et al. 2005; Bayard De Volo et al. 2008). To increase DNA yield we heated buffer AE (elution buffer) at 70 °C for 10 min before use. Buffer AE contains the organic compound Tris [tris(hydroxymethyl) aminomethane, $(HOCH_2)_3CNH_2$)] and EDTA [Ethylenediaminetetraacetic acid ($C_{10}H_{16}N_2O_8$)] which functions to rehydrate the nucleic acids and release DNA from the silica membrane. This process is improved when the DNA and silica are exposed to higher temperatures (Bruns et al. 2007; Zhou and Ling 2011; Haddad et al. 2017). We used an elution volume of 100 µl in a two-step process, giving a final volume of 200 µl which was subsequently stored at − 20 °C. Although using half the recommended volume of elution buffer decreases the DNA yield, the aim was to increase the final concentration of DNA as it is well documented that a higher concentration of starting DNA in PCR reactions improves the likelihood of successful DNA amplification (Kishore et al. 2006; Rohland and Hofreiter 2007; Graziano et al. 2013).

In order to assess whether the alterations to the protocol had optimised DNA concentration following extraction, we compared the concentration of DNA from samples extracted using the standard manufacturer's protocol and the protocol we have outlined above. DNA concentrations for samples extracted using the standard protocol were obtained from a feather set that arrived in the laboratory in 2016 (n = 50). We used a number generator to randomly select 20 plumulaceous feather samples from the feather set obtained in 2016 and extracted using the standard protocol and from the feather set obtained in 2018 and extracted using the optimised extraction method. The DNA concentration of each sample was quantified using the Invitrogen™ Qubit™ 3.0 Fluorometer, which has a DNA detection range of 0.50–600 ng/ml, using dsDNA High Sensitivity settings following the manufacturer's protocol (Table 1). A Shapiro–Wilk test (Shapiro and Francia 1972) indicated that the data were not normally distributed ($p < 0.05$) therefore a Wilcox test (Wilcox 2008) to compare the difference in DNA extracted when using the standard protocol and the optimised protocol outlined in this study. All analyses were performed in R (R Core Team 2013).The results of the test showed that samples that underwent the optimised protocol had a significantly higher extracted DNA concentration than the standard protocol ($W = 81$, p value < 0.01, Cohens's $d = 0.89$). Samples that were lower than the detection range of the Invitrogen™ Qubit™ 3.0 Fluorometer were assigned the value 0.49 ng/ml. Table 1 shows that 30% of the samples extracted using the standard protocol were of too low concentration to evaluate. In comparison, only 5% of the samples that were extracted using the optimised protocol had a concentration below the range of the Invitrogen™ Qubit™ 3.0 Fluorometer. Therefore, both DNA extraction success rate and DNA concentration were increased by the optimised protocol.

Table 1 Concentration of DNA (ng/ml) from samples extracted using the standard QIAGEN user developed DNA extraction protocol and samples extracted using the optimised extraction protocol

Standard protocol		Optimised protocol	
2016 Samples	DNA conc. (ng/ml)	2018 Samples	DNA conc. (ng/ml)
P2	< 0.50[a]	M31	7.45
P4	26.9	M40	10.8
P5	< 0.50	M44	1.13
P7	13.0	M46	5.18
P8	< 0.50[a]	M63	3.60
P12	< 0.50[a]	M68	4.60
M1	6.09	M83	1.76
M2	1.28	M95	217.67
M5	0.56	M100	58.17
M8	0.81	M108	1.33
M18	0.82	M114	50.7
M19	7.92	M115	4.64
M22	1.74	M118	< 0.50[a]
M23	1.35	P37	32.1
M28	15.9	P38	307.33
F3	1.32	P39	4.50
F4	1.23	P45	45.43
F5	0.51	P49	34.13
F6	< 0.50[a]	P69	2.23
F8	< 0.50[a]	F12	1.87
Mean	4.1185	Mean	39.7555
Standard error	± 1.56	Standard error	± 17.82
Median	1.03	Median	4.91

[a]DNA concentration was too low to read and therefore out of range of the Invitrogen™ Qubit™ 3.0 Fluorometer (range 0.50–600 ng/ml) using dsDNA High sensitivity settings

To test that target DNA, rather than that of subsidiary material, had been extracted and to ascertain the potential presence of PCR inhibitors, which are common when using non-invasively collected samples (Waits and Paetkau 2005; von Thaden et al. 2017), avian cytochrome b (cyt b), was amplified. We designed primers using Primer3 (Koressaar and Remm 2007; Untergasser et al. 2012) based on a cytochrome b sequence from the white-tipped dove (*Leptotila verreauxi*), whose whole mitochondrial genome is available on GenBank, accession number: NC_015190.1 (Pacheco et al. 2011). The Primer3 output for the designed primers is provided in Online Resource 1. This species was chosen as it has been suggested, based on the ecology of the species, that it is closely related to the Grenada dove (Blockstein and Hardy 1989), although molecular phylogenetic analysis has not yet been carried out for the Grenada dove. The primers were designed to include a 200–250 bp region of the cyt b gene, with a length of around 20 bases long, a GC content of around 50% and melting temperatures that are no more than 5 °C apart (Dieffenbach et al. 1993; Naqib et al. 2019). The chosen primers targeted a 203 bp region of cytochrome b gene: CYTB_2 Forward: 5′-CTGCCTACT AACCCAGATCCT-3′ and CYTB_2 Reverse: 5′-AGGAGC CGTAGTAGAGTCCT-3′. To prevent contamination of samples, PCR was conducted in a PCR hood where tube racks, pipettes and tubes were exposed to UV light for 20 min prior to setting up the reaction (Bayard De Volo et al. 2008). PCR was conducted using illustra™ PuReTaq Ready-To-Go™ PCR Beads (GE HEALTHCARE; Chicargo) with a final volume of 25 µl containing ~2.5 units of recombinant PuReTaq DNA polymerase, 200 µM of dNTP's in 10 mM Tris–HCl, 50 mM KCl and 1.5 mM $MgCl_2$ stabilizers, BSA and reaction buffer, 5 µl of DNA template and a negative control using sterile H_2O. PCR was conducted on a TECHNE TC-3000 thermocycler (Bibby Scientific Ltd; Stone) using the following conditions optimised for this primer set: initial denaturation at 95 °C for 5 min, 45 cycles of 95 °C for 30 s, 48 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 5 min. PCR products were separated by electrophoresis (Westermeier 2005) on a 2% (Mardis and McCombie 2017) agarose gel (Thermo Fisher Scientific, Waltham) and visualised using a BioRad Gel Doc™ EZ Imager and Image lab 4.0 software (Bio-Rad Laboratories 2017). DNA extraction using the optimised method resulted in a visible band on the gel at the 203 bp target region of avian cyt b for 154 out of 158 (97.5%) of the feathers being used in this study (Table 2) thus confirming target DNA rather than subsidiary material had been amplified.

Table 2 Number of successful and failed amplifications of a 203 bp target region of avian cytochrome b gene, recorded by feather type, following DNA extraction using our optimised technique (prior to including the primerless PCR step)

Feather type	Number of feathers	Number of successful amplifications	Number of failed amplifications
Secondary	3	2 (66.7%)	1 (33.3%)
Primary	3	3 (100%)	0 (0%)
Plumulaceous	152	149 (98%)	3(2%)
Total	158	154 (97.5%)	4 (2.5%)

Amplification was considered successful if a visible band was present on a 2% agarose gel for the target region

Primerless PCR

Primerless PCR, also known as 'DNA shuffling' or 'sexual PCR', exposes the DNA template to Taq DNA polymerase, dNTPs and a heating and cooling cycle which serves to denature the sample into smaller fragments which then anneal to each other (Stemmer 1994; Melnikov and Youngman 1999; Brakmann and Schwienhorst 2004; Suenaga et al. 2005; An et al. 2011). This is known as self-priming and functions to repair DNA damage such as nicks, fragmentation, abasic sites and blocked 3′-ends in degraded DNA samples that may inhibit amplification (Diegoli et al. 2012).

Primerless PCR reactions were conducted using illustra™ PuReTaq Ready-To-Go™ PCR Beads with a final volume of 25 µl including 5 µl of DNA template. Samples were subjected to a PCR cycle with the cycling parameters: initial denaturation at 95 °C for 5 min, 10 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 5 min. A negative control was also generated at this stage by exposing 25 µl of PCR reaction mix and no DNA template to the same PCR cycle to ensure no contamination or false amplification occurred during the primerless PCR process.

In order to investigate the efficacy of the primerless PCR process we chose low quality and quantity samples for comparison. The samples chosen were identified by lack of gel band presence, brightness and/or clarity following optimised DNA extraction and amplification (Jacobs et al. 2013; Thiel et al. 2014) of the 203 bp region of cytochrome b gene outlined above. Samples consisted of: 10 plumulaceous feathers, one secondary feather, and one egg shell. Each sample was used as a substrate for the amplification of the barcoding region of the cytochrome oxidase I gene using primer set AWCF1 and AWCintR2 (C1; 328 bp) (Patel et al. 2010), as described in the small amplicon strategy section below, with and without a prior primerless PCR stage. A standardised dilution factor was used to ensure the same amount of DNA template was used in each primered PCR reaction. Following amplification, the samples were visualised on the UV transilluminator as a comparison for effective amplification with and without exposure to primerless PCR. This can be seen

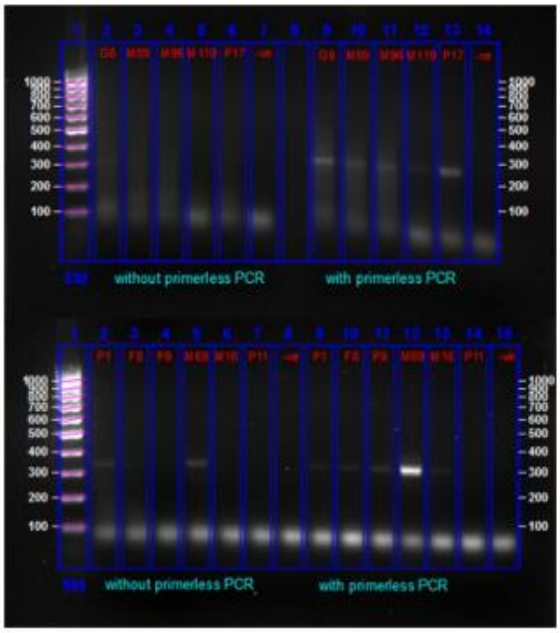

Fig. 2 Gel image showing the amplification of non-invasively collected plumulaceous feather samples [with the exception one secondary feather (G8)] using primer set AWCF1 and AWCintR2 (328 bp) (Patel et al. 2010) both with and without exposure to primerless PCR

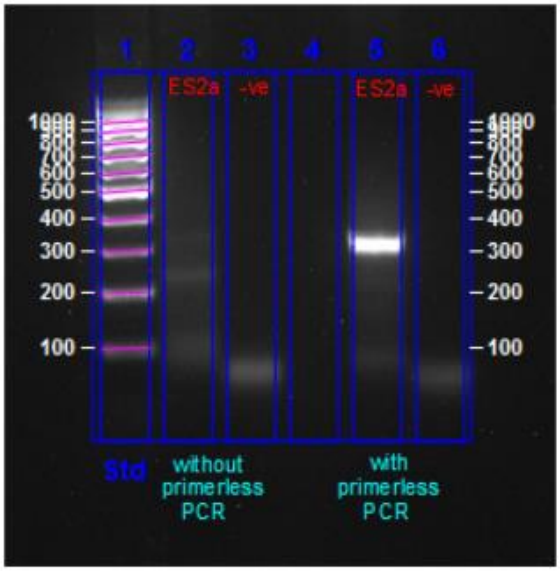

Fig. 3 Gel image showing the amplification of a non-invasively collected egg shell sample using primer set AWCF1 and AWCintR2 (328 bp) (Patel et al. 2010) both with and without exposure to primerless PCR

for feather samples in Fig. 2 and the egg shell sample in Fig. 3, which show that more bands were present and were more defined after the addition of the primerless PCR step. Nine of the twelve samples failed to amplify without the addition of primerless PCR but successfully amplified and presented clear bands when exposed to primerless PCR. For example, sample 69 (Fig. 2) shows a brighter and more defined band is present after under-going the primerless PCR process. The increased prevalence of bands and the improved clarity, brightness and definition of the present bands after primerless PCR indicates the improved quality of DNA after exposure to this process.

Small amplicon strategy

The third step we adopted was a small amplicon strategy to successfully amplify and sequence three small overlapping amplicons, which were combined to construct a longer and more informative section of the gene. Small amplicons have an increased likelihood of amplification (Broquet et al. 2006; Fischer et al. 2016; Debode et al. 2017) thus we targeted 200–250 bp amplicons (Rohland and Hofreiter 2007; Stiller et al. 2009). We performed primered PCR on our samples, following inclusion of the primerless PCR step, along with a primerless negative control and a standard negative control, using the following overlapping primer sets, which are known to amplify *Columbiforme* COI barcoding region: AWCF1 and AWCintR2 (C1; 328 bp), AWCintF2 and AWCintR4 (C2; 314 bp), AWCintF4 and AWCR6 (C3; 350 bp) (Patel et al. 2010). The PCR reaction was subjected to the same cycling parameters as used in the primerless PCR stage: initial denaturation at 95 °C for 5 min, 10 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 5 min. PCR products were separated and visualised using agarose-gel electrophoresis. All three primer sets successfully amplified DNA from our non-invasively collected samples (Fig. 4). Samples were purified using the QIAquick PCR Purification as per the manufacturer's protocol (QIAGEN Inc., Crawley). Samples were then prepared for sequencing using the Mix2Seq Kit (Eurofins Genomics, Luxembourg) according to the manufacturer's instructions and submitted to Eurofins Genomics, where they were sequenced using Sanger sequencing methods.

Sequence data for the three overlapping regions were reconstructed to create an approximately 650 bp sequence (Fig. 5a). Firstly, consensus sequences were obtained for each amplicon by aligning the forward and reverse sequence data in NCBI Basic Local Alignment Search Tool (BLAST®) (Johnson et al. 2008) and were used in all further reconstructions and alignments. Obtaining consensus sequence is particularly important when using primerless PCR as random fragmentation and self-priming

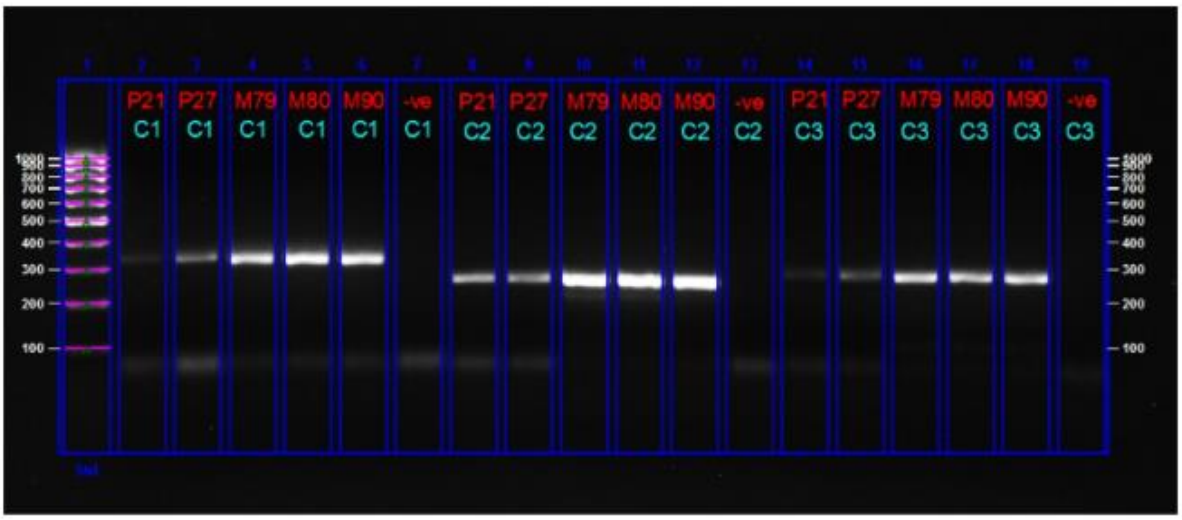

Fig. 4 Gel image showing successful amplification of the three small amplicons using primer sets AWCF1 and AWCintR2 (C1; 328 bp), AWCintF2 and AWCintR4 (C2; 314 bp), AWCintF4 and AWCR6 (C3; 350 bp) (Patel et al. 2010) from moulted plumulaceous feathers collected non-invasively and opportunistically in the field

can introduce artificial recombinants or base errors but this can be counteracted by using consensus sequence (Weber et al. 2000). To assemble the longer combined sequence, each of the small amplicons were aligned using a global alignment algorithm with Emboss Explorer merger tool (Fig. 5b). A heuristic approach is used to select the base with the best local sequence quality score in the case of any mismatches (Bell and Kramvis 2013). Highly degraded DNA can produce poor read length or low quality base calls in the sequence data, particularly at the ends of the trace producing "messy" end sequences (Bell and Kramvis 2013). Therefore, most probably due to the highly degraded nature of the samples used in this study, some base inconsistencies were recognised. In these cases the Emboss Merger local quality score, along with the electropherogram obtained from Eurofins for each sequence (Fig. 5c), were assessed and the base with the highest quality score as per both the emboss and Eurofins sequence quality assessment was selected (Bell and Kramvis 2013). Due to the non-invasive sample collection method used for these samples, along with the inability to identify the species from the morphological features of the feather alone, each sequence was run in NCBI's Basic Local Alignment Search Tool (BLAST®) to predict species identification (Johnson et al. 2008). This search indicated that the sample, presented in Fig. 5, was from the Caribbean columbid, white-crowned pigeon (*Patagioenas leucocephala*), with a BLAST total score of 989 and a query coverage of 91% to Genbank sequence JJQ175689.1 (Schindel et al. 2011). Combined sequence data obtained from the three small amplicons were aligned with the corresponding COI barcoding region sequence data from a known *L. wellsi* specimen (unpublished data—Genbank reference sequence not yet available for this species) and *P. leucocephala* (accession number JQ175689.1) (Schindel et al. 2011) to confirm the expected nitrogenous base positions (Johnson et al. 2008), which is particularly important given the possibility of potential base errors introduced by primerless PCR (Weber et al. 2000). Target-species was confirmed for the 650 bp length of the COI barcoding region from non-invasively collected plumulaceous feather samples.

Discussion

The methods outlined in this study: an optimised user-developed DNA extraction protocol, use of a primerless PCR technique, and a small amplicon PCR strategy, facilitated the attainment of target-species mtDNA sequence data of 650 bp in length from non-invasively collected plumulaceous feather samples. Problems arising due to poor quality of DNA extracted from non-invasively collected samples can often dissuade researchers from proceeding with genetic analysis, causing the potential of such samples to go unrecognised (Horváth et al. 2005; Speller et al. 2011). This can also lead to a preference in invasive as opposed to non-invasive sample collection methods due to the higher confidence in invasive sample quality (Johnson and Clayton 2000a; Harvey et al. 2006). The methods we have outlined allow such samples, which previously may have been discounted for genetic analysis, to be successfully used to obtain informative sequence data.

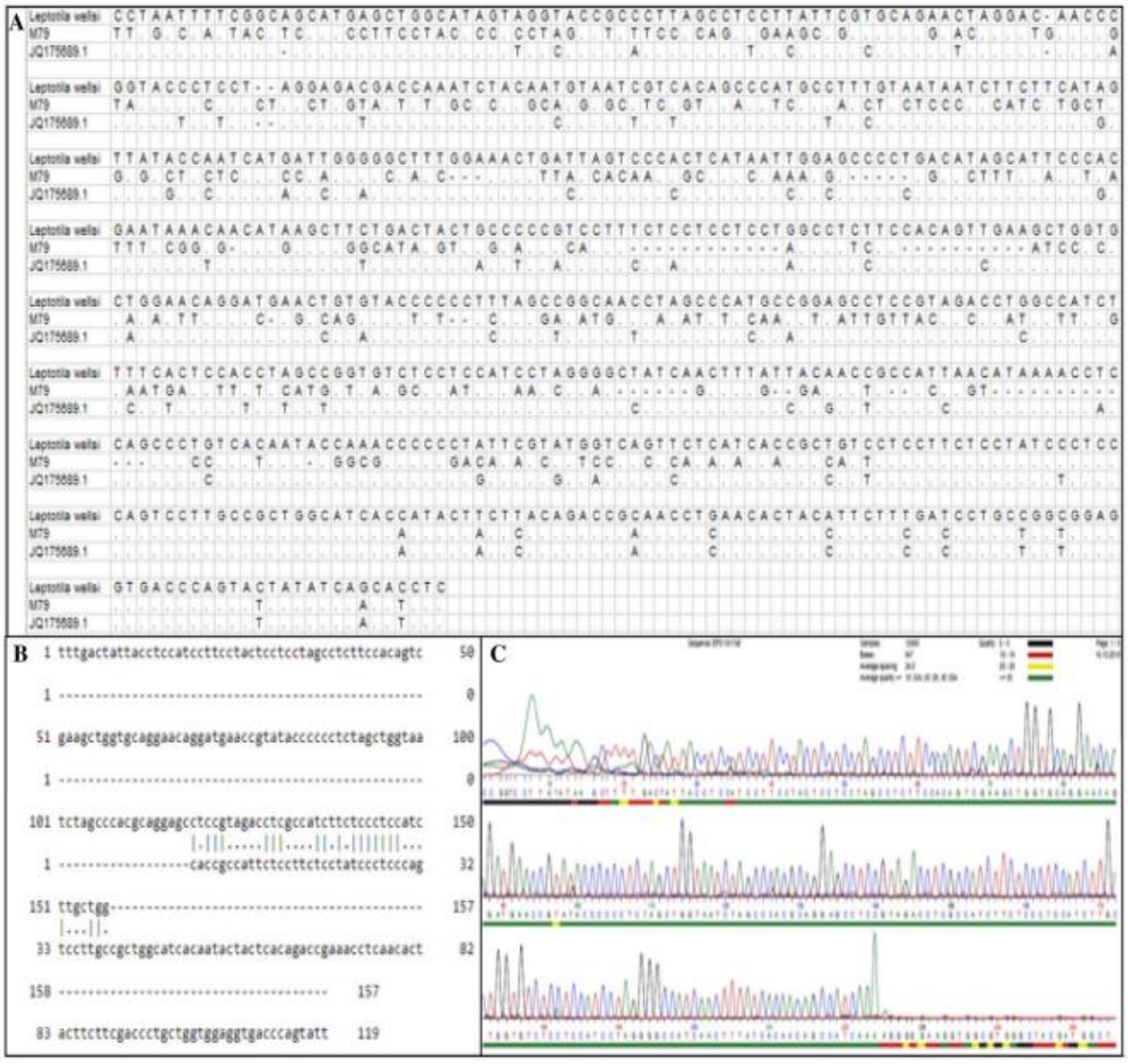

Fig. 5 Sequence data from sample M79. **a** The 650 bp sequence obtained for sample M79 aligned with the corresponding COI barcoding region sequence data for *Leptotila wellsi* and *Patagioenas leucocephala,* labelled with its accession number JQ175689.1 (Schindel et al. 2011). **b** Emboss Explorer (Bell and Kramvis 2013) OUTPUT example for two overlapping sequences from sample M79 using forward and reverse consensus sequences. **c** Electropherogram example with base quality score as assigned by Eurofins for the forward sequence of COI_2

It is widely documented that feather type influences the success and yield of DNA extraction from feather samples (Bayard De Volo et al. 2008; Hogan et al. 2008). Primary feathers are widely used as a resourse for genetic monitoring of wild bird populations, as they often contain a blood clot located in the superior umbilicus of the feather shaft (Horváth et al. 2005). Along with assessing the genetic variation within populations (Nichols et al. 2001), feather samples have been used to investigate phylogeny and biogeography (Johnson and Clayton 2000b), and to understand the impact of anthropological processes on genetic structure and gene flow (Fourcade et al. 2016) of bird species. However, a study on molecular sexing and microsatellite genotyping of hyacinth macaw (*Anodorhynchus hyacinthinus*) found that despite using larger moulted feathers (with a size greater than 20 cm) than in this study, feathers that spent more than 7 days in the field and were of poor physical quality, had a low success rate in yielding sufficient DNA for genetic analysis (Presti et al. 2013). Nevertheless, we have demonstrated that plumulaceous feathers that are found in the field

do not necessarily need to be excluded from sample sets as they can still be utilised to yield informative data.

The use of primerless PCR increased the amplification success in subsequent PCR reactions for samples that had proven difficult to amplify. Bands from samples exposed to primerless PCR were visually clearer and brighter, which suggests a higher quality of DNA (Hughes-Stamm et al. 2011; Jacobs et al. 2013; Lucena-Aguilar et al. 2016). Primerless PCR has been used in studies of ancient DNA, including that of Weber et al. (2000), who increased the successful amplification of ancient DNA from bone samples when investigating the population bottleneck of the northern elephant seal (Weber et al. 2000). The same technique was adopted using museum samples from the African horseshoe bat (*Rhinolophus darlingi*) to investigate the phenotypic convergence of its evolutionary history (Jacobs et al. 2013). Both studies reported that primerless PCR improved the recovery of DNA from ancient samples but did not comment on differences in DNA quality post primerless PCR (Weber 2004; Jacobs et al. 2013). To the best of our knowledge, our study is the first to demonstrate the utility of primerless PCR to increase DNA quality for degraded non-invasive feather samples and demonstrates that this technique can be applied to a variety of non-invasive samples collected in the field.

Although the primerless PCR technique recovers the quality of DNA obtained from the degraded samples this process does not completely repair damaged DNA, particularly when the DNA damage is highly fragmented, which is a common problem with samples kept in non-optimal conditions (Diegoli et al. 2012). The varying quality of sequence data at ends of the trace producing "messy" end sequences is a particular problem for overlapping regions resulting in base inconsistencies (Stiller et al. 2009). However, this is not specific to the techniques we describe here and is prevalent in many genetics datasets, though it is not widely documented (Sobel et al. 2002; Hackett and Broadfoot 2003; Bonin et al. 2004). Sequencing error can lead to misidentification of individuals, misinterpretations and erroneous conclusions to be drawn from genetic analysis of relatedness and population structure (Hogan et al. 2008). Sequencing error can occur at any stage when obtaining genetic information but there are a number of actions that can be taken to minimise these errors (Bonin et al. 2004). The following have been adopted in this study and are proposed for future studies to limit the risk of sequencing error following primerless PCR: (1) the amplification of small amplicons because genotyping error correlates with amplicon size (Vili et al. 2013); (2) the inclusion of negative controls (Waits and Paetkau 2005; Alda et al. 2007; Boonseub et al. 2012); (3) sequence quality assurance by using only forward and reverse consensus sequences with further analysis and inspection of electropherogram scoring levels; and (4) cross-reading and aligning sequence data with reference samples (Weber et al. 2000; Bonin et al. 2004). For downsteam analyses, it is also recommended to include a sequencing error rate when using genetic data for population and phylogenetic analyses (Bonin et al. 2004).

We have outlined methods that achieve successful extraction and amplification of an informative length of mtDNA from non-invasively collected plumulaceous feather samples. The analysis of mtDNA has many practical applications in conservation including species identification as well as presence/absence detection. The high mutation rate of mtDNA in comparison to nDNA allows the identification of Evolutionarily Significant Units (ESUs), giving an insight into the phylogenetics of a species (Cronin 1993; Wan et al. 2004; Gupta and Bhardwaj 2013). Data pertaining to phylogenetics have multiple conservation implications including evolutionary divergence and speciation, along with phylogeography and rates of change relating to morphology and behaviour of a species (Huang et al. 2009). Determining the point at which speciation occurs or defining a species as genetically distinct—known as the phylogenetic species concept—is particularly useful for resolving taxonomic uncertainties, outlining wildlife legislation, and in identifying conservation priorities (Hazevoet 1996; Wan et al. 2004; Pellens and Grandcolas 2016; Chen et al. 2018). Analysis of mtDNA can be used to make long-term species conservation action plans from an evolutionary perspective (Nielsen et al. 2017) and to inform short-term demographic management of populations through identification of population change and connectivity therefore providing information on the effects of habitat fragmentation (Cronin 1993; Moritz 1994; Nabholz et al. 2009). Therefore, our improved methods of extraction and amplification of mtDNA from non-invasively collected, low quality feather samples, have the potential to extend the applicability of molecular analyses in studies aimed at the conservation of endangered bird species, for which it is typically difficult to obtain high quality samples.

Conclusion

In conclusion, the optimised user-developed DNA extraction protocol, along with the use of the primerless PCR technique, and a small amplicon PCR strategy, are sufficient to enable DNA extraction and mtDNA amplification from moulted plumulaceous feathers collected non-invasively and opportunistically in the field. This not only provides evidence in support of using non-invasive sampling methods for genetic analyses, in particular when applied to endangered species, but also highlights the utility of biological material kept in non-optimal conditions, may previously have been discounted (Rawlence et al. 2009; Vili et al. 2013). Data collected in this manner is informative for species identification, presence/absence detection, population structure and

phylogenetic analyses of rare and elusive species (Bonin et al. 2004; Marucco et al. 2011; Adam et al. 2014), all of which are key questions in conservation research.

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Appendix IV: Life history trait data for 24 Neotropical columbid species

Species Name	Clutch size n	Clutches per year	Incubation (days)	Fledging age (days)	Adult body mass (grams)	Female body mass (grams)	Male body mass (grams)	No sex body mass (grams)	Generation length (years)	Male maturity (days)	Female maturity (days)	Hatching weight (grams)	Longevity (years)
Columba livia	2	4	17.5	31	320	270	307	337	5.8	140	140	15.2	21.5
Columbina inca	2	4	14	15	48.75	NA	NA	47.5	4.3	NA	NA	NA	7.8
Columbina minuta	2	NA	13.5	13	32.9	31	30.8	32.9	4.3	NA	NA	NA	NA
Columbina passerina	2	2.5	13.25	11	35	34.5	33.1	34	4.3	75	75	5	7.2
Columbina picui	2	NA	NA	NA	47	50	NA	46.5	4.3	NA	NA	NA	NA
Columbina squammata	2	NA	NA	NA	52.45	52	NA	52.9	4.3	NA	NA	NA	NA
Columbina talpacoti	2	2.5	12.5	115	46.375	46.2	46.3	46.25	4.3	NA	NA	NA	NA
Leptotila cassini	2	NA	NA	NA	157.25	NA	NA	157.25	4.2	NA	NA	NA	NA
Leptotila megalura	2	NA	NA	NA	218	NA	NA	218	4.2	NA	NA	NA	NA
Leptotila plumbeiceps	2	NA	14	15	171	NA	NA	171	4.2	NA	NA	NA	NA
Leptotila rufaxilla	1.7	NA	NA	NA	154.3333333	139	153	160	4.2	NA	NA	NA	NA
Leptotila verreauxi	2	1	14	16	147.5	120.5	126.5	153	4.2	NA	NA	NA	8.6
Leptotila wellsi	NA	NA	NA	NA	200	NA	NA	200	NA	NA	NA	NA	NA
Patagioenas fasciata	1.075	2	19	30	352.5	357	375.75	340	6.9	365	492.9625	NA	18.5
Patagioenas leucocephala	1.5	NA	14	21	247	233.35	245.45	194	6	365	365	NA	14.4
Patagioenas plumbea	1	NA	13.8	21	173.5	184.75	170.5	NA	6.6	NA	NA	NA	NA
Patagioenas speciosa	1.5	NA	NA	16.5	262	304	293.5	302	6.6	NA	NA	NA	NA
Streptopelia decaocto	2	4	15	17	154.7	167.5	174.5	193	5.3	365	364.234	NA	17.7
Streptopelia roseogrisea	2	NA	13.5	15	153.5	148	165	153.5	5.3	NA	NA	NA	12
Zenaida asiatica	2	2	13.25	15	153	153	NA	153	7.1	365	365	7.7	21.8
Zenaida auriculata	2	NA	14	NA	110.75	95	112	111.7	6.8	NA	NA	NA	NA
Zenaida aurita	2	NA	14	14	159	NA	NA	159	6.8	300	330	NA	6.3
Zenaida galapagoensis	2	NA	14	15	113.075	NA	NA	113.075	6.8	NA	NA	NA	NA
Zenaida macroura	2	4	14.4	14	119	115.5	123	123	6.7	80	136.3125	5	19.3
HBW Alive (2020) Handbook of the Birds of the World Alive | HBW Alive. Lynx Edicions													
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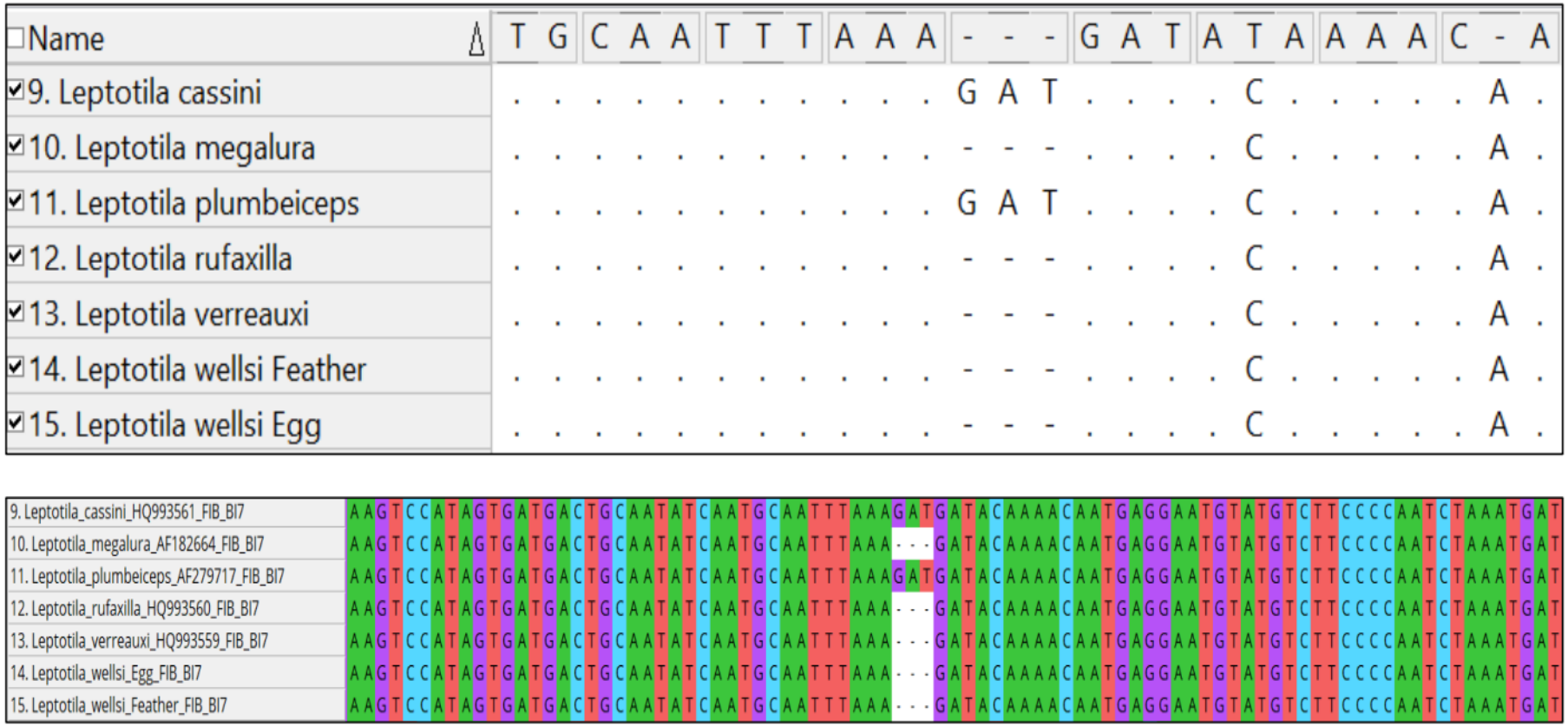
Myhrvold NP, Baldridge E, Chan B, et al (2015) An amniote life-history database to perform comparative analyses with birds, mammals, and reptiles. Ecology 96:3109–000. doi: 10.1890/15-0846r.1													

Appendix V: Alignment of the nuclear β-fibrinogen gene for *Leptotila* species showing the indel present in *Leptotila plumbeiceps* and *Leptotila cassinii* sequences. Conserved regions are denoted by • and variable regions denoted by -. Alignments of β-fibrinogen intron 7 sequences were constructed using the method outlined in Prychitko and Moore 2003.



Appendix VI: Phylogenetic signal lambda (λ) value per life history trait

Life History Trait	λ value
litter_or_clutch_size_n	1.03537452
litters_or_clutches_per_y	7.61E-05
incubation_days	0.05452824
fledging_age_days	6.84E-05
adult_body_mass_g	1.0246996
female_body_mass_g	1.02162865
male_body_mass_g	1.02560503
no_sex_body_mass_g	1.00869828
Generation_length_years	1.03537452
male_maturity_d	4.84E-05
female_maturity_d	0.32784191
longevity_y	6.98E-05
birth_or_hatching_weight_g	7.80E-05

Appendix VII: Published version of Chapter 4

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Phylogenetic placement and life history trait imputation for Grenada Dove *Leptotila wellsi*

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Abstract

Phylogenetic analyses can be used to resolve taxonomic uncertainties and reconstruct a species' evolutionary history. This can be combined with ecological data to predict missing life history traits which are important for creation of conservation management strategies. We investigated the evolutionary and life history of the 'Critically Endangered' Grenada Dove *Leptotila wellsi* by estimating its phylogenetic placement and using this new phylogeny to test the accuracy of phylogenetic comparative methods for estimating both documented and unknown life history traits. We extracted DNA from two Grenada Dove samples and obtained sequences from three mitochondrial markers: Cytochrome oxidase I (*COI*), NADH dehydrogenase 2 (*ND2*) and Cytochrome b (*Cyt b*); and one nuclear marker: β-Fibrinogen intron 7 (*β-FIB*). We present the first genetic data obtained for the Grenada Dove. Our data identify the Grey-Chested Dove *Leptotila cassinii* as the species which shares both a most recent common ancestor, with an estimated divergence of approximately 2.53 million years ago, and the smallest genetic distance ($P = 0.0303$) with the Grenada Dove. Life history trait values for the Grenada Dove predicted from our analyses using phylogenetic imputation are: clutch size = 2 (± 0.09) eggs; clutches per year = 1.4 (± 0.81); incubation time = 14.2 (± 0.75) days; hatching weight = 3.8 g (± 1.05) and single imputation: fledging age (genus median) = 15.5 days, longevity (genus median) = 8.6 years. This study contributes novel information regarding evolutionary history and life history characteristics to inform long-term conservation actions for a 'Critically Endangered' species.

Introduction

Over 12% of extant bird species are threatened with extinction (Brooks *et al.* 2008), 58.8% of which are island species (Tershy *et al.* 2015). Natural extinction rates are higher in island populations than in mainland populations. Sensitivity to environmental change, predation by introduced predators, and small population sizes are all common characteristics among island avifaunas; therefore, island endemic species are at an increased risk of extinction (Frankham 1998). Taxonomic patterns in extinction risk suggest that families containing high numbers of endemic species are more susceptible to extinction (Lockwood *et al.* 2000). Resolution of unknown taxonomic and evolutionary relationships can therefore contribute to a greater understanding of extinction vulnerabilities among bird groups (Lockwood *et al.* 2000, Johnson *et al.* 2007).

Advances in systematic biology have allowed phylogenetic information to be combined with phenotypic data to further infer the evolutionary processes, based on the assumption that phenotypic traits within groups of species will be influenced by shared ancestry (Cressler *et al.* 2015, Bastide *et al.* 2018). Recently, comparative phylogenetic methods for missing data imputation have been employed to predict physiological adaptations, conservation status, and functional life history traits for data deficient species (Riek and Bruggeman 2013, Swenson 2014, González-del-Pliego *et al.* 2019, James *et al.* 2021). Predictive models are particularly useful for estimation of trait values when species are rare and elusive, locations are remote, and fiscal constraints limit field surveys (Wood *et al.* 2018, Horswill *et al.* 2021). Life history traits such as generation time, longevity, male and female maturity, clutch size, and incubation and fledging times can be used to evaluate the capability of a species to adapt to climate change and habitat fragmentation as well as being essential to estimate population trends (Mace *et al.* 2008, Pearson *et al.* 2014, Storchová and Hořák 2018, Cuervo and Møller 2019, Horswill *et al.* 2021).

Pigeons and doves (Columbiformes; Columbidae) are one of the oldest lineages of birds, inhabiting a wide range of habitats on six of the seven continents, despite which their evolutionary history is still poorly resolved (Pereira *et al.* 2007, Soares *et al.* 2016). Neotropical columbids may form the most ancient lineage (Johnson and Clayton 2000b, Shapiro *et al.*

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2002) but have been little studied, with fundamental taxonomy, conservation status, ecological and life-history data still largely unknown (Brooks *et al.* 2008, Devenish-Nelson *et al.* 2019). Therefore, gathering information about Neotropical species is essential and urgent for conservation of this diverse group of birds (Latta 2012).

The Neotropical columbid genus *Leptotila* comprises 11 morphologically similar species, of which one, the Grenada Dove *L. wellsi*, is 'Critically Endangered' (BirdLife International 2018). Endemic to Grenada in the eastern Caribbean Sea, the Grenada Dove is found in two seemingly isolated populations in the west and south-west of the island (Rusk *et al.* 2008, Rusk 2017). The most recent population assessment estimated that 160 ± 30 individuals remain of this species and showed a decreasing population trajectory (Rivera-Milán *et al.* 2015, BirdLife International 2018). Important details about the species' biology, such as longevity and incubation period, are currently unreported. The Grenada Dove was once considered a subspecies of the Grey-fronted Dove *L. rufaxilla* until reclassified as a distinct species based on morphological and acoustic evidence (Blockstein and Hardy 1989). However, its genetic distinctiveness and relationships have never been investigated. Anecdotal evidence indicates that the Grenada Dove is closely related to the Caribbean Dove *L. jamaicensis* due to morphological similarities (Anthony Jeremiah, Head of Forest and National Parks Department, Government of Grenada, pers. comm.).

By investigating both the evolutionary and life history of the Grenada Dove, and by estimating its phylogenetic placement, we were able to predict currently unreported life history traits. We extracted DNA from Grenada Dove samples and obtained sequences from three mitochondrial DNA markers: Cytochrome oxidase I (*COI*), NADH dehydrogenase 2 (*ND2*) and Cytochrome b (*Cyt b*); and one nuclear marker: β-Fibrinogen intron 7 (*β-FIB*). We used these markers first to infer the evolutionary history of this species amongst other Neotropical columbid species. We then used phylogenetic comparative methods for missing data imputation, based on this evolutionary history, to predict unknown life history trait values to inform conservation management decisions.

Methods

DNA Extraction and PCR

Non-invasively collected eggshell and feather samples ($n = 2$) were collected in 2018 and 2017, respectively. Samples were transported by airmail to the UK in June 2018 where they were cleaned with 70% ethanol and stored at −20°C. DNA barcoding was used to confirm species identification for the samples using methods outlined in Patel *et al.* (2010). As this is the first time Grenada dove DNA has been obtained, there is no reference available for this species. However, as it is the only member of its genus resident on Grenada, where all of our samples were collected, we conducted a BLAST® search with the resulting data and found that sequences obtained for this study were consistent with previously obtained sequences for *Leptotila*. We used the optimised method for DNA extraction, using the QIAGEN DNeasy® Blood and Tissue kit and the primerless PCR protocol, outlined in Peters *et al.* (2019).

The substrate from the primerless PCR process was used to amplify the mitochondrial markers *Cyt b* (882 bp), *COI* (613 bp) and *ND2* (949 bp) and the nuclear marker *β-FIB* (902 bp). Primer sequences and the corresponding annealing temperatures are given in Supporting Information 1. PCR cycling parameters for all except the *COI* region were: initial denaturation at 95°C for 5 min, 45 cycles of 95°C for 30 s, 50–60°C (primer specific, see Appendix S1 in the online supplementary material) for 30 s, 72°C for 60 s and a final extension at 72°C for 5 min; and for the *COI* region: 95°C for 5 min, 10 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 60 s, 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 60 s and a final extension at 72°C for 5 min. Where regions could not be amplified using a single primer set, additional primers were designed using Primer3 (Untergasser *et al.* 2012) allowing a small amplicon strategy to be used where regions were amplified in smaller overlapping sections (as per Peters *et al.* 2019).

Prior to sequencing, samples were purified as per the manufacturer's protocol using the QIAquick PCR Purification kit (QIAGEN Inc., Crawley). Preparation and submission of samples were carried out according to the Eurofins Genomics guidelines for the Mix2Seq kit (Eurofins Genomics, Luxembourg) where sequencing was performed using Sanger sequencing methods. As the primerless PCR technique has the potential to increase the chance of sequencing error, amplification and sequencing were performed in triplicate in order to obtain consensus data for each region (Peters *et al.* 2019, Weber *et al.* 2000). Sequence reconstruction and alignments were conducted using Sequencher 5.4.6 (Gene Codes Corporation 2017).

Phylogenetics

Sequences from 24 Neotropical columbids with Common Crane *Grus grus* as an outgroup were obtained for all markers from Genbank (Benson *et al.* 2013; Table 1). Phylogenetic tree construction was performed using MEGAX 10.2.4 (Kumar *et al.* 2018). We used Maximum Likelihood to fit 24 different nucleotide substitution models and used Akaike information criterion (*AIC*) and Bayesian information criterion (*BIC*) to select the best models (Schwarz 1978, Akaike 1987, Nei and Kumar 2000).

The Hasegawa-Kishino-Yano (*HKY*) model using a discrete Gamma distribution (+*G*) was the best-supported model for the combined mitochondrial and nuclear DNA dataset of 3,346 bp and thus evolutionary analyses were performed using this model with 500 bootstrap replicates (Hasegawa *et al.* 1985). In order to check the consistency of this tree we also built the tree using the *BEAST approach implemented in BEAST v2.6.6 (Heled and Drummond 2010, Bouckaert *et al.* 2019) and the resulting species tree provided the same topology as the concatenated tree (for details see Appendix S2). As mtDNA tends to have a larger proportion of variable bases than nDNA (Allio *et al.* 2017), we also inferred the position of the Grenada Dove within the *Leptotila* genus based on the mtDNA and nDNA separately, to provide a comparison between the two marker types, using the following best-supported models as per *AIC* and *BIC*: General Time Reversible model ([+I], 38.01% sites), for a total of 2,444 bp of mtDNA (Tavaré 1986), and Tamura 3-parameter model, for a total of 902 bp of nDNA (Nei and Kumar 2000). We also inferred the position of the Grenada Dove in relation to *L. jamaicensis*, using a reduced genetic dataset using the Hasegawa-Kishino-Yano model (+G, parameter = 0.2496) for a total of 2,720 bp of *ND2, Cyt b* and *β-FIB*.

Time-calibrated phylogenies were reconstructed to infer molecular-based estimates of divergence by applying the RelTime-ML method following the protocol outlined in Mello (2018). Fossil calibrations are widely used for molecular dating, but no fossil records are available for the taxa used in this study (Ksepka *et al.* 2015, Peters and McClennen 2015). We therefore used confidence intervals of minimum and maximum boundary estimations from the TimeTree database, which provides

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Table 1. List of species and their Genbank accession number for all sequences used in phylogenetic analysis.

Species	Complete mtDNA genome	*β-FIB*	COI	ND2	Cyt b
Colimbina squammata	N/A	AF182651.1[4]	EF373368.1[16]	KJ645747.1[25]	AF483347.1[21]
Columba livia	KF926376.1[11]	AY082415.1[17]	N/A	N/A	N/A
Columbina inca	N/A	AF182650.1[4]	DQ433527.1[8]	KJ645733.1[25]	AF182683.1[4]
Columbina minuta	N/A	AF182652.1[4]	JQ174506.1[20]	KJ645743.1[25]	KJ639100.1[25]
Columbina passerina	N/A	AF182653.1[4]	JN801583.1[26]	KJ645740.1[25]	KF924046.1[19]
Columbina picui	N/A	AF182654.1[4]	FJ027422.1[9]	KJ645737.1[25]	KJ639094.1[25]
Columbina talpacoti	N/A	KJ668681.1[25]	FJ027429.1[9]	KJ645744.1[25]	KJ639101.1[25]
Grus grus	FJ769849.1[10]	DQ881960.1[2]	N/A	N/A	N/A
Leptotila cassinii	N/A	HQ993561.1[6]	JQ175250.1[20]	FJ175707.1[12]	HQ993505.1[6]
Leptotila jamaicensis	NA	AF279706.1[1]	NA	HQ993543.1[6]	AF279716.1[1]
Leptotila megalura	N/A	AF182664.1[3]	FJ027741.1[9]	HQ993545.1[6]	AF483342.1[21]
Leptotila plumbeiceps	N/A	AF279717.1[1]	JQ175252.1[20]	HQ993544.1[6]	AF279707.1[21]
Leptotila rufaxilla	N/A	HQ993560.1[6]	JQ175255.1[20]	AF251546.1[4]	HQ993504.1[6]
Leptotila verreauxi	HM640214.1[15]	HQ993559.1[6]	N/A	N/A	N/A
Patagioenas fasciata	KX902239.1[22]	AF353465.1[5]	N/A	N/A	N/A
Patagioenas leucocephala	N/A	AF182656.1[4]	JQ175689.1[20]	AY274070.1[23]	AF182689.1[4]
Patagioenas plumbea	N/A	AF182658[4]	JQ175696.1[20]	AF251547[4]	AF182691[4]
Patagioenas speciosa	N/A	AF279721[1]	JQ175700.1[20]	AF353442[5]	AF279711[1]
Streptopelia decaocto	NC_037513.1[18]	AF353449.1[5]	N/A	N/A	N/A
Streptopelia roseogrisea	N/A	AF353450[5]	JN801382.1[24]	AF353419[5]	AF353399[5]
Zenaida asiatica	N/A	AF258324.1[4]	DQ433271.1[7]	AF251543.1[4]	AF251533.1[3]
Zenaida auriculata	HM640211.1[15]	AF182667.1[4]	N/A	N/A	N/A
Zenaida aurita	N/A	AF258323.1[3]	JN639032.1[14]	AF251542.1[4]	AF182704.1[4]
Zenaida galapagoensis	N/A	AF258322.1[4]	JQ420133.1[13]	AF251539.1[4]	AF251531.1[3]
Zenaida macroura	NC_031863.1[22]	AY082416.1[17]	N/A	N/A	N/A

Sources: [1](Clayton *et al.* 2003); [2](Ericson *et al.* 2006); [3](Johnson & Clayton, 2000a); [4](Johnson & Clayton, 2000b); [5](Johnson *et al.*, 2001); [6](Johnson & Weckstein, 2011); [7](Kerr *et al.* 2006); [8](Kerr *et al.* 2007); [9](Kerr *et al.* 2009); [10](Krajewski *et al.* 2010); [11](Li *et al.* 2015); [12](Miller *et al.* 2008); [13](Monceau *et al.* 2012); [14](Monceau *et al.* 2013); [15](Pacheco *et al.* 2011); [16](Pereira *et al.* 2007); [17](Prychitko and Moore 2003); [18](Qu 2018); [19](Santiago-Alarcon *et al.* 2014); [20](Schindel *et al.* 2011); [21](Shapiro *et al.* 2002); [22](Soares *et al.* 2016); [23](Sorenson *et al.* 2003); [24](Stoeckle 2011); [25](Sweet and Johnson 2015); [26](Tavares *et al.* 2011)

divergence time estimates from published studies integrated with a geological timescale, to calibrate divergence time (Kumar *et al.* 2017, Mello 2018, Tamura *et al.* 2012, 2018).

Life history trait imputation

To predict life history traits for the Grenada Dove we used multivariate phylogenetic comparative methods for missing data imputation implemented in the *Rphylopars* package version 0.3.0 (Goolsby *et al.* 2017) in *R* version 3.6.2 (R Core Team 2020). This approach uses a multivariate procedure of phylogenetic covariance across species to predict differences in life history traits (Goolsby *et al.* 2017, James *et al.* 2020). We imputed phylogenetic covariance across 24 Neotropical columbid species. Twelve life history traits - clutch size, clutches per year, incubation time, hatching weight, fledging age, longevity, generation length, male and female maturity, no-sex body mass (unreported sex; Myhrvold *et al.* 2015), and male and female body mass - were obtained for each of these species, where available, from primary and grey literature (Appendix S3; Myhrvold *et al.* 2015, HBW Alive 2020).

Available life history trait values for the Grenada Dove were a clutch size of two eggs (Rusk, unpubl. data), an imputed generation length of 4.2 years calculated using the IUCN generation length calculator (BirdLife International 2018), a fledging age of 14 days from focal nest observations and an estimate of two or three nesting attempts per year (Rusk, unpubl. data). We imputed these traits to determine whether multivariate phylogenetic comparative methods generated similar values. We fitted evolutionary models and used *AIC* and *BIC* as well as assessing model parameters to select the best fitting model. Phylogenetic signals were calculated using a maximum-likelihood approach to quantify the relationship between life history trait values and phylogenetic signal in order to ascertain model precision per life history trait (Penone *et al.* 2014, James *et al.* 2021). We tested the accuracy of the *RPhylopars* method by predicting known values, which we removed individually from the dataset. We focused on the genus *Columbina* as this genus contained the most known data, which allowed us to sequentially remove a total of 20% of these known values for each trait with minimal decrease in the accuracy of predictions (Penone *et al.* 2014). We compared the predicted values from *RPhylopars* to the

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true value to assess the accuracy of predictions. We also calculated the median values, used in some life-history imputation without explicit phylogenetic information (e.g. Bird *et al.* 2020), for fledging age and longevity.

Results

Phylogenetics

There were no polymorphisms observed between the two Grenada Dove sequences. There were 27 nucleotide polymorphisms across 3,346 bp that were unique to the Grenada Dove and not present in any other *Leptotila* species used in this investigation, consisting of 21 transition mutations, two transversion mutations and one transition/transversion mutation (depending on the *Leptotila* species) in the mtDNA and three transitional mutations in the nDNA β-FIB gene. The phylogenetic tree constructed using concatenated sequence data - *Cyt b* (882 bp), *COI* (613 bp), *ND2* (949 bp), and *β-FIB* (902 bp) - for Neotropical columbids indicates that the Grenada Dove falls within the monophyletic group formed by *Leptotila* species (Figure 1a.). The Grenada Dove shared a most recent common ancestor with its sister group containing *L. cassinii* and *L. plumbeiceps* approximately 2.53 million years ago (mya) (Figure 2a). The smallest genetic distance ($P = 0.0303$) where 100 nucleotides were polymorphic (Table 2) was identified between the Grenada Dove and *L. cassinii*. The phylogenetic tree built with the reduced data and containing *L. jamaicensis* (Figure 3) still shows a sister relationship between the Grenada Dove and *L. cassinii* and *L. plumbeiceps*.

The mitochondrial phylogenetic trees shown in Figures 1b and 2b suggests that the Grenada Dove shared its most recent common ancestor with *L. cassinii* and *L. plumbeiceps* approximately 2.18 mya, which is consistent with the phylogenetic tree constructed with the concatenated data set in Figure 1a. The nuclear phylogenetic trees shown in Figures 1c and 2c suggest that the Grenada Dove shared its most recent common ancestor with *L. megalura* and *L. ruffaxilla* approximately 2.75 mya, dissimilar to the most recent common ancestor shown by the phylogenetic trees constructed with the combined (Figure 1a and 2a) and mtDNA (Figure 1b and 2b) datasets. Analysis of the *β-FIB* gene relevels an indel (Appendix S4) in *L. cassinii* and *L. plumbeiceps* sequences which is not shared by the Grenada Dove or any of the other *Leptotila* species included in this study.

Life history trait imputation

Strong phylogenetic signals were revealed for clutch size, no-sex body mass, and male and female body mass, whereas weak phylogenetic signal was detected for clutches per year, incubation time, hatching weight, fledging age, longevity, and generation length (Appendix S5). Model validation (Figure 4) showed lower variation relative to each trait around seven of the twelve life history trait

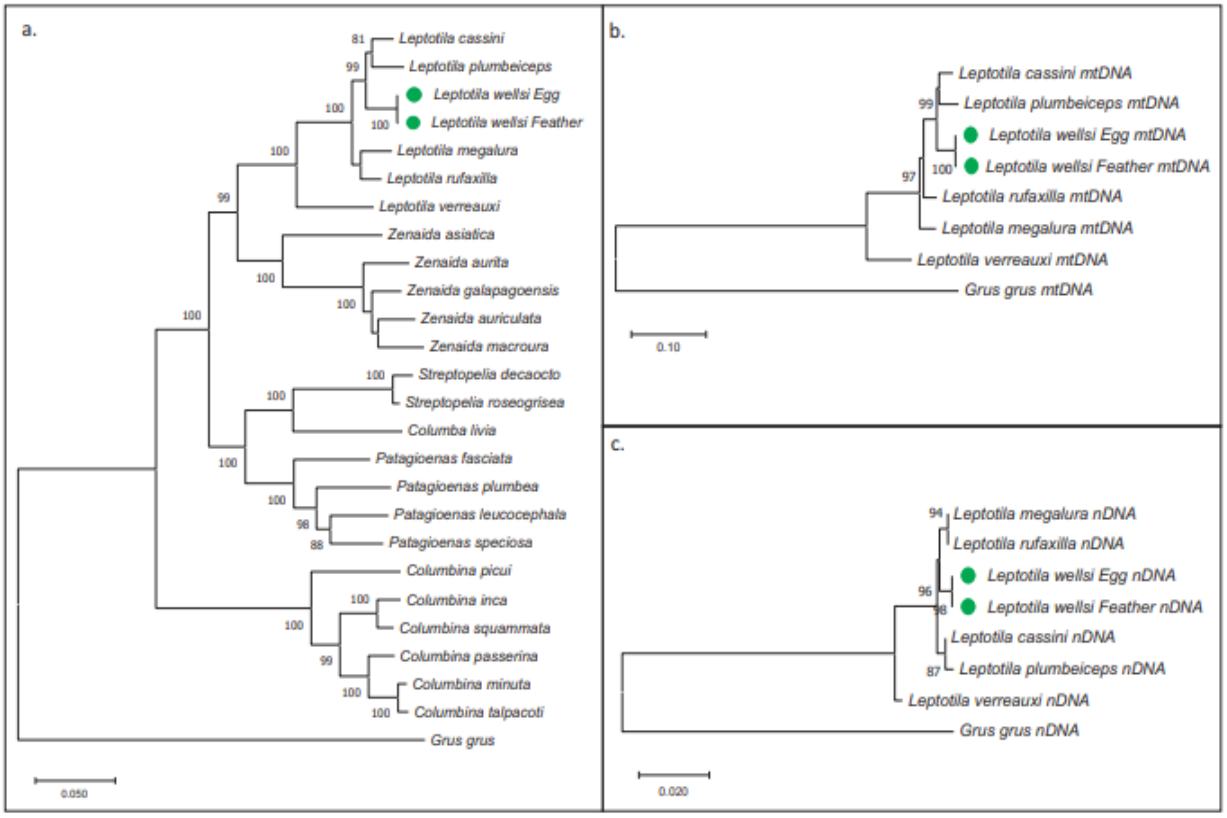

Figure 1. Phylogenetic analysis for 24 Neotropical columbid species and outgroup using the Maximum Likelihood method. Grenada Dove sequences indicated by the circular symbol; a. all mitochondrial and nuclear sequence data estimated using the Hasegawa-Kishino-Yano model (+G, parameter = 0.2281); b. mtDNA using General Time Reversible model ([+I], 38.01% sites); c. β-fibrinogen using the Tamura 3-parameter model. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site and nodes supported by bootstrap values of >80%.

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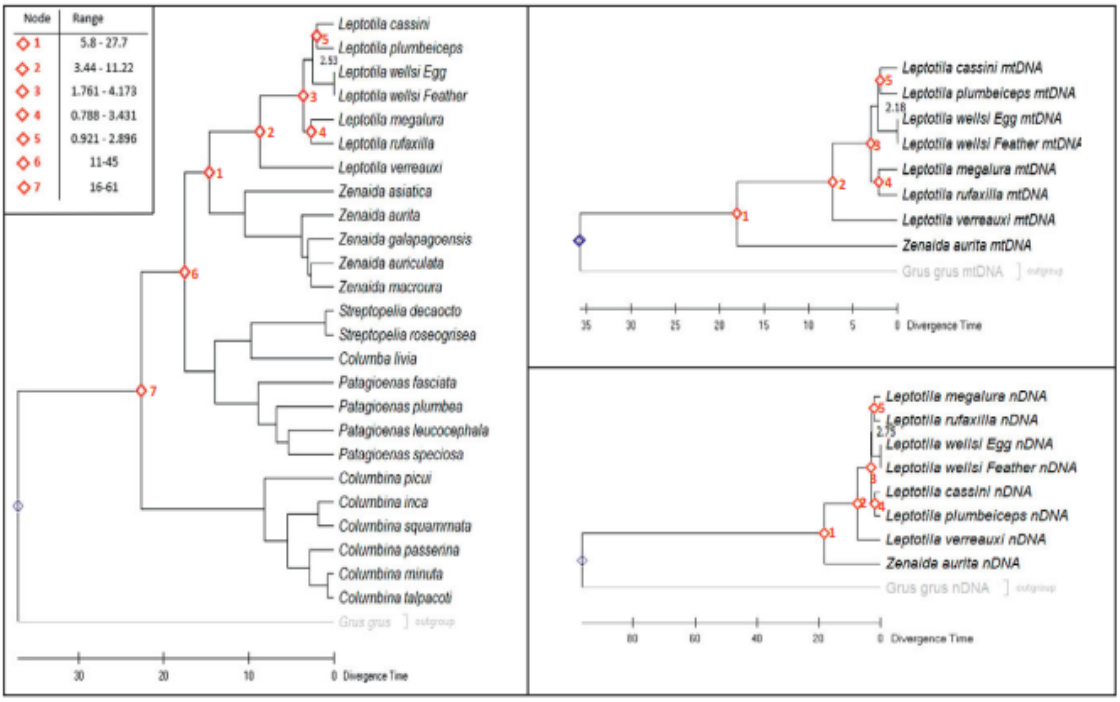

Figure 2. Time-calibrated phylogenies for *Leptotila* species and outgroup using the RelTime-ML method a. all mitochondrial and nuclear sequence data estimated using the Hasegawa-Kishino-Yano model (+G, parameter = 0.1547), b. mtDNA General Time Reversible model ([+I], 35.97% sites) c. β-fibrinogen using the Tamura 3-parameter model. Number scale indicates million years ago (mya). Nodes indicate 7 estimated divergence time calibration points of estimated divergence time ranges in million years ago (mya) obtained using the TimeTree resource (Kumar *et al.* 2017).

Table 2. The number of substitutions per site (p-distance) and the number of base differences (polymorphic sites) between sequences for a total of 3,346 bp positions in the final dataset. *2,720 bp positions in the final dataset.

Species	P - distance	Number of polymorphic sites
Leptotila cassinii	0.0303	100
Leptotila megalura	0.0424	140
Leptotila plumbeiceps	0.0339	112
Leptotila verreauxi	0.0719	237
Leptotila rufaxilla	0.0383	126
*Leptotila jamaicensis**	0.0622	167

predictions including clutch size, clutches per year, incubation time, hatching weight, fledging age, longevity, and generation length and were therefore considered accurate. However, the remaining five life history trait predictions including male and female maturity, no-sex body mass, and male and female body mass showed a high level of variation from the true value and therefore were discounted. Despite suggested trait accuracy from the model validation for fledging age and longevity, we report large standard error values in addition to weak phylogenetic signal, and as such, genus medians were considered a more accurate representation for these traits. Known values for generation length for *Leptotila* are themselves imputed using the IUCN generation length calculator, and thus we discounted this trait from our results. Overall, six life history trait values were predicted for the Grenada Dove with confidence (Figure 5) using multivariate and single imputation procedures: a. clutch size = 2 (±0.09 SE) eggs, b. clutches per year = 1.4 (±0.81 *S.E.*), c. incubation time=14.2 (±0.75 SE) days. d. hatching weight = 3.8 g (± 1.05 SE) grams, fledging age = 15.5 days (genus median) days, f. longevity = 8.6 years (genus median).

Discussion

This study reports the first genetic data obtained for the Grenada Dove and supports a sister relationship with Grey-chested Dove *L. cassinii* along with Grey-headed Dove *L. plumbeiceps*, whose ranges span Central America into Colombia (BirdLife International 2021). We provide estimations for six previously unpublished life history traits for the Grenada Dove some of which, such as clutch size and clutches per year, have been found to be correlated with extinction risk (Parlato *et al.* 2015).

Leptotila cassinii was the species with the smallest genetic distance from the Grenada Dove ($P = 0.0303$) with 100 bp nucleotide polymorphisms based on concatenated sequence data and phylogenetic reconstruction. The most recent common ancestor was shared approximately 2.53 mya between *L. cassinii*, *L. plumbeiceps*, and the Grenada Dove. However, when the mtDNA and nDNA are considered separately our results show disparity. The mtDNA shows the same relationship displayed by the concatenated data described above, but nDNA analysis revealed that the most recent common ancestor was shared between *L. rufaxilla*, *L. megalura*, and the Grenada Dove. The mito-nuclear incongruence may have arisen for a variety of reasons; a larger amount of mtDNA sequence data was used providing a stronger phylogenetic signal, the

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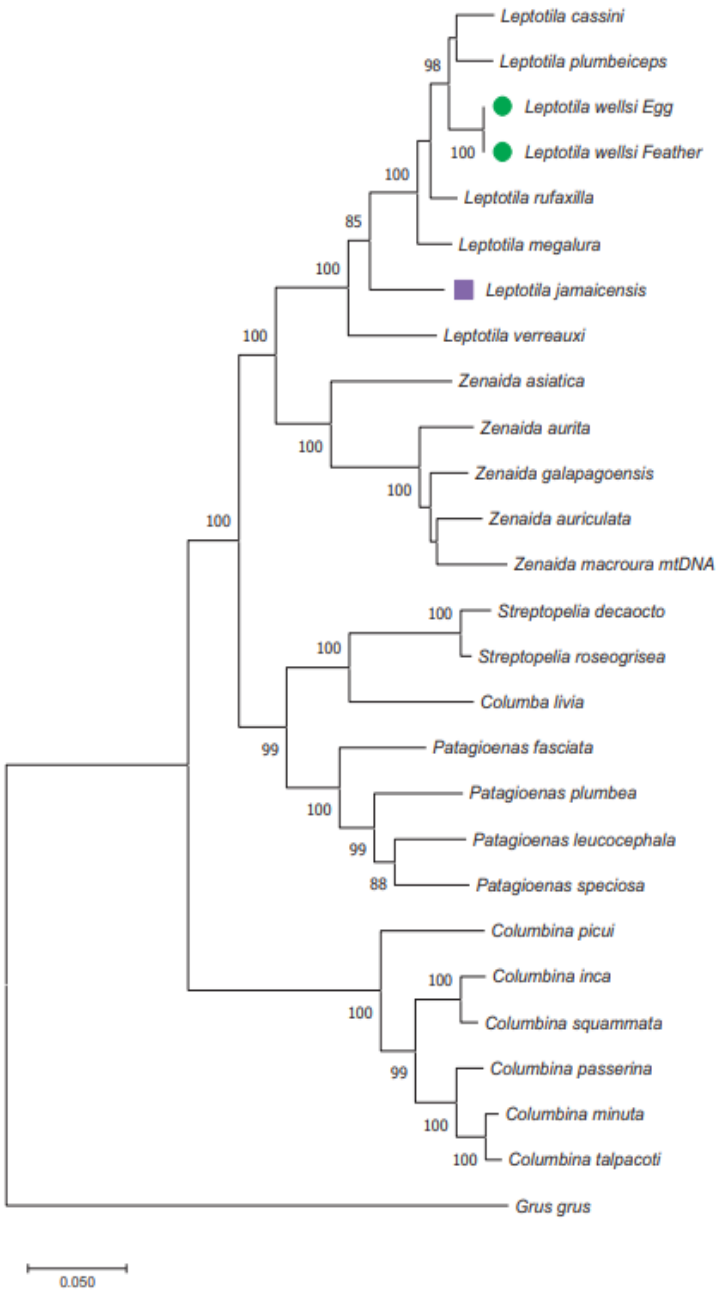

Figure 3. Phylogenetic analysis for 25 Neotropical columbid species and outgroup using the Maximum Likelihood method. Grenada Dove sequences indicated by the circular symbol and *Leptotila jamaicensis* are indicated by a square using the Hasegawa-Kishino-Yano model (+G, parameter = 0.2496). Tree drawn to scale, with branch lengths measured in the number of substitutions per site and nodes supported by bootstrap values of >80%.

differing characteristics between these markers such as differential lineage sorting and linkage disequilibrium, all of which can lead to ambiguous patterns of variation and evolutionary inferences (Hurst and Jiggins 2005, Rubinoff and Holland 2005). Overall, we identify *L. cassinii* and *L. plumbeiceps* as the species sharing the most recent common ancestor with the Grenada Dove, however, we recommend caution due to the disparity between the mitochondrial or nuclear phylogeny. Nevertheless, our result was unexpected as *L. cassinii* is predominantly a Central American species and has a larger geographical distance from Grenada than *L. rufaxilla*, of which the Grenada Dove was previously thought to be a subspecies. We reveal a genetic difference ($P = 0.04$) and 126 nucleotide polymorphisms between the Grenada Dove and *L. rufaxilla*. Our findings support the work of Blockstein and Hardy (1989) whose morphological analysis (showing differing morphological traits such as cinnamon underwing on primary feathers, a greater extent

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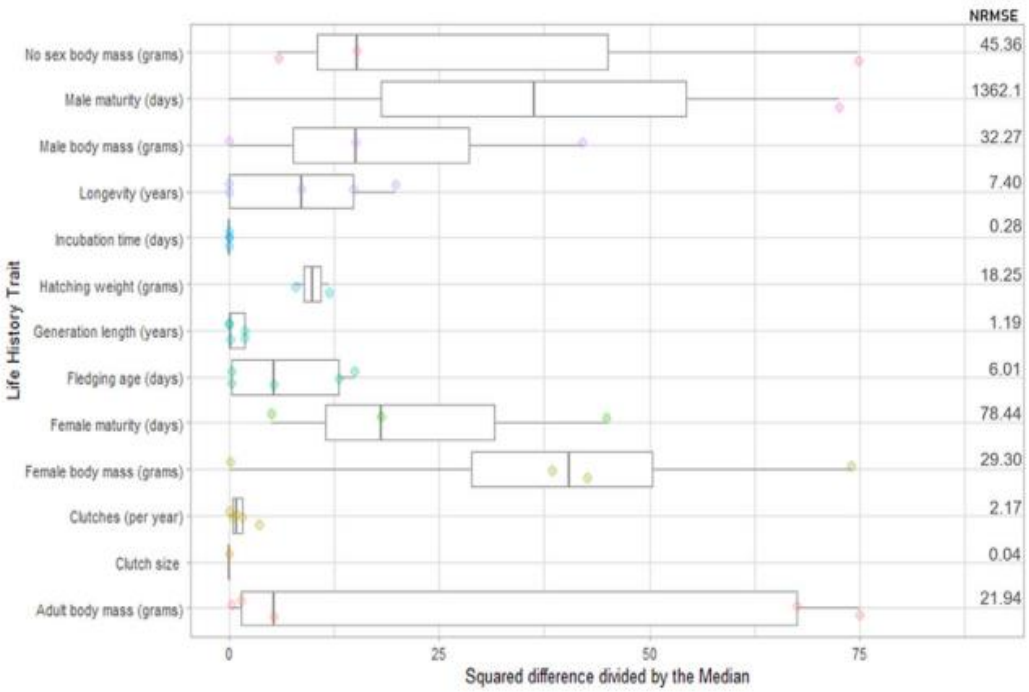

Figure 4. Plot displaying the squared difference between the eb model predicted value and the true value divided by the trait median using the genus Columbina along with Normalized Root Mean Squared Error [NRMSE (Penone *et al.* 2014, James *et al.* 2021)]. Raw data are represented by the " and the first, second and third quartile are represented by the box plot. Mean Squared Error per life history trait.

of white abdomen and a lesser extent of white on the tail tips) and sonographic data (no response was made by the Grenada Dove to *L. rufaxilla* playback presentations) led to the reclassification of the Grenada Dove as a distinct taxon. Anecdotal evidence suggested the Grenada Dove is closely related to *L. jamaicensis* (Anthony Jeremiah, Head of Forest and National Parks Department, Government of Grenada, pers. comm.), however our results show that the Grenada Dove shares common ancestors with four other *Leptotila* more recently than its shared ancestor with *L. jamaicensis* and with a greater genetic distance and number of nucleotide polymorphisms in comparison.

The evolutionary history of the *Leptotila* genus was inferred by Johnson and Weckstein (2011) using a molecular phylogeny to investigate the role of the Central American land-bridge in avian dispersal-driven diversification events. Their results show that this genus diverged into two clades at a time that coincides with the closure of the Isthmus of Panama forming the Central American land-bridge. Their study suggests that the northern clade dispersed southwards from North America stepwise through Central America, crossing the land-bridge into South America, while the southern clade, containing *L. rufaxilla* and *L. cassinii*, dispersed in the opposite direction from South America across the Panama Isthmus (Johnson and Weckstein 2011). Our results indicate that the Grenada Dove is placed within the southern clade and not with the northern clade containing *L. jamaicensis*. We hypothesise that there was a shared evolutionary history with an upwards dispersal event from South America by the ancestor shared by *L. rufaxilla*, *L. cassinii* and the Grenada Dove with a range expansion to Central America and the southern islands of the Lesser Antilles. Dispersal over the barriers presented by the South American Andes and the Caribbean Sea implies reduced gene flow between the ancestral populations and eventual isolation. This is supported by the absence of the indel in the Grenada Dove nDNA, suggesting this mutation occurred after divergence from the shared common ancestor with *L. cassinii* and *L. plumbeiceps*. Our data support the hypothesis that allopatric speciation due to isolation of the Grenada Dove and exposure to differing selective pressures resulted in the evolution of Grenada Dove as a distinct species.

Our analyses were able to predict six life history values for the Grenada Dove. Values for clutch size, clutches per year and fledging age agreed with documented values from focal observations (Rusk, unpubl. data). With our model estimations of only two eggs per clutch and 1.4 clutches per year the Grenada Dove has a relatively slow breeding rate, which has been shown to be correlated with extinction risk (Robinson *et al.* 2010, Hutchings *et al.* 2013). Given that IUCN generated a 4.2 year generation length and with a low predicted longevity (genus median = 8.6 years), these values suggest this species would be slow to recover after a major population decline (Bird *et al.* 2020). As not all traits are phylogenetically conserved (Kamilar and Cooper 2013), we tested for phylogenetic signal and found that signal strength varied amongst traits. Clutch size and generation length both exhibited strong phylogenetic signals, as is expected owing to their correlation with body mass, which indicates phylogenetic conservatism of these life history traits (Kamilar and Cooper 2013, Calhoon *et al.* 2014). All other traits exhibited low phylogenetic signal strength, which is not uncommon in traits such as these that often evolve as a response to differing environmental conditions (Kamilar and Cooper 2013, Martin *et al.* 2018). Although our model validation has suggested that many of our predicted values are accurate, these results have to

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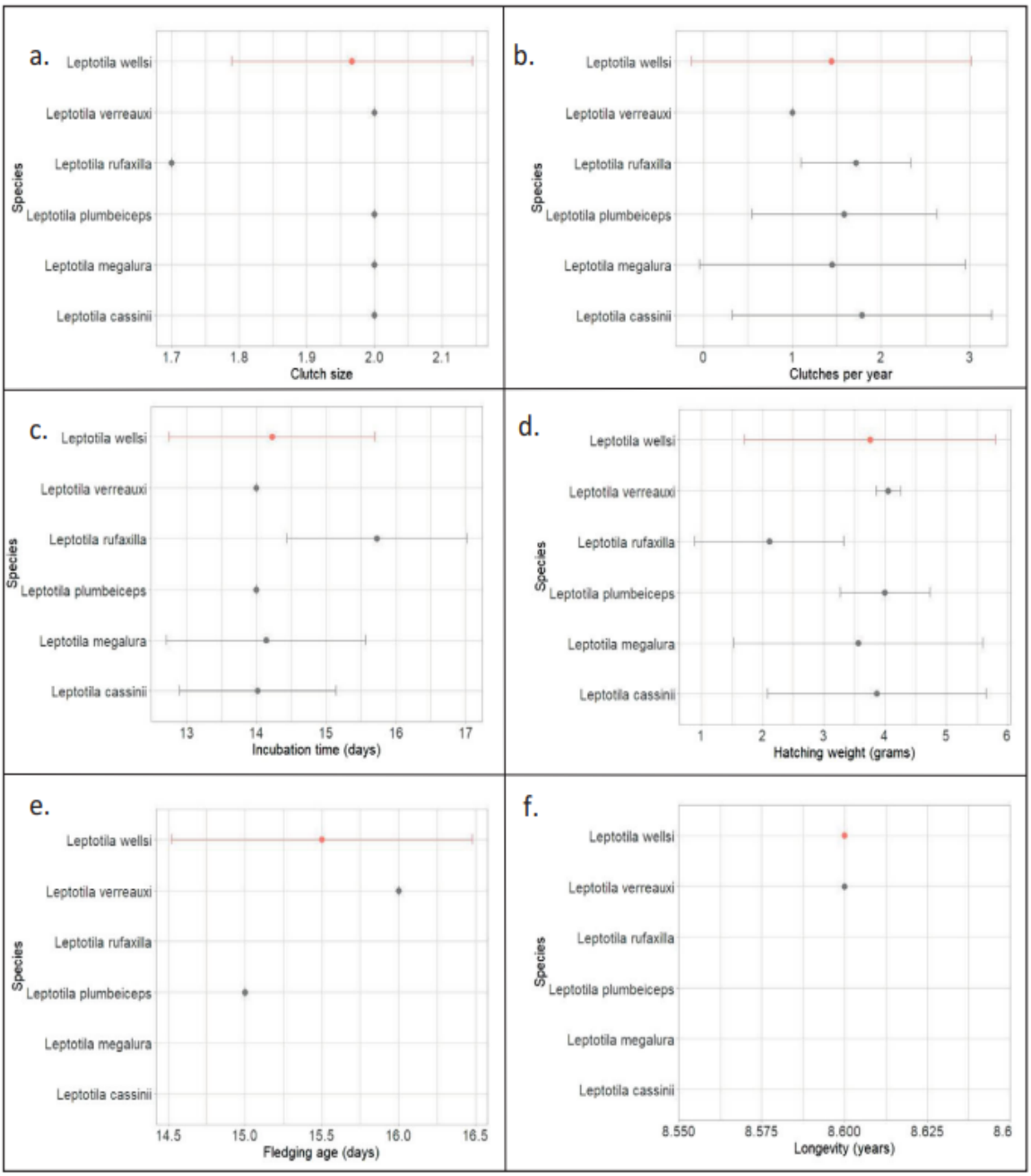

Figure 5. Predicted trait values with upper and lower 95% confidence intervals for the Grenada Dove *Leptotila wellsi* in comparison with other Leptotila species for the following lift history traits using the EB model: a. clutch size, b. clutches per year, c. incubation time, d. hatching weight; and median estimates: e. fledging age, f. longevity. Species of interest highlighted red. Known trait values represented by individual grey point.

be used cautiously, and would benefit from validation from field data (Ando 2019, Bolton *et al.* 2016).

When comparing predicted values for life history traits for the Grenada Dove and *L. cassinii* (the species we reveal shares the most recent common ancestor and the smallest genetic distance) and *L. rufaxilla* (the species it was once considered a subspecies of) we find that a clutch size of two and more than one clutch per year were shared between all three species. The Grenada Dove and *L. cassinii* both having a hatching weight of over 3 g and an incubation time of around 14 days whereas *L. rufaxilla* has a slightly lower hatching weight of around 2 g and a slightly higher incubation time of 15 days. While there is no great difference in life history traits between studied members of the *Leptotila* genus, as expected, we find that the species with life history traits most similar to the Grenada Dove is *L. cassinii* which is the species we also reveal has the smallest genetic distance.

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The more favourable conservation status of the other *Leptotila* species examined in this study may be attributed to their much larger ranges and availability of suitable habitat (BirdLife International 2021). We reinforce the need for increasing suitable habitat available to the Grenada Dove to reduce extinction risk, both through habitat restoration and protection, as has been outlined in the recovery and action plan for this species, as well as identification of additional habitat for establishing new populations and enabling population increase (Rusk *et al.* 2008).

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Supplementary Materials. To view supplementary material for this article, please visit http://doi.org/10.1017/S0959270922000065.

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Supplementary Material

Phylogenetic placement and life history trait imputation for Grenada Dove *Leptotila wellsi*

CATHERINE PETERS, MATTHEW GEARY, HOWARD P. NELSON, BONNIE L. RUSK, ACHAZ VON HARDENBERG and ANNA MUIR

Contents

Appendix S1. List of primers and primer sequences used in this study along with the specific annealing temperature and product sizes.

Gene	Primer	Sequence (5'->3')	T_a (°C)	Amplicon	Reference
COI	AWCF1[1]	CGC YTW AAC AYT CYG CCA TCT TAC C	50*	328bps	(Patel *et al.* 2010)
COI	AWCR2	ATG TTG TTT ATG AGT GGG AAT GCT ATG	50*		
COI	AWCF2	ATC GGA GCC CCA GAC ATA GCA TT	50*	314bps	
COI	AWCR4	TTG ATG GCT GTT GTG ATA AAG TTG AT	50*		
COI	AWCF4	TCC TCA ATC CTG GGA GCA ATC AAC TT	50*	352bps	
COI	AW CR6j	ATT CCT ATG TAG CCG AAT GGT TCT TT	50*		
Cyt b	L14841	AAA AGC TTC CAT CCA ACA TCT CAG CAT GAT GAA A	54	1042bps	(Kocher et al. 1989)
Cyt b	H4a	AAG TGG TAA GTC TTC AGT CTT TGG TTT ACA AGA CC	54		(Harshman
Cyt b	CYTB 32 F	ACC CAG ATC YTA ACA GGY CT	55	350bps	Designed by this study
Cyt b	CYTB 32 R	GTT TGG CCG ATG TAG GGG AT	55		
Cyt b	CYTB 316 F	GTG CCA CAG TCA TCA CCA AC	55	358bps	
Cyt b	CYTB 316 R	GGG TGT AAA GTT TTC TGG GTC T	55		
Cyt b	CYTB 585 F	AAG ACA TCC TCG GCT TCA CA	55	362bps	
Cyt b	CYTB 585 F	GTA GGT GAG GGA GGC AAG TT	55		
ND2	L5216	GGC CCA TAC CCC GRA AAT	60	550bps	(Sorenson 2003)
ND2	H5766	RGA KGA GAA RGC YAG GAT YTT KCG	60		
ND2	L5758	GGC TGA ATR GGM CTN AAY CAR AC	54	555bps	
ND2	H6131	CTCTTATTTAAGGCTTTGAAGGC	54		
ND2	ND2 420 F	CTG CCC TGC TAC TCT CAA CT	55	408bps	Designed by this study
ND2	ND2 420 R	GGT GAG TTC TTG GAT GAT GAG	55		
FIB B I7	FIB-BI7L	GGA GAA AAC AGG ACA ATG AC A ATT CAC	50	1000bp	(Prychitko and

FIB B I7	FIB-BI7U	TCC CCA GTA GTA TCT GCC ATT A GG GTT	50		Moore
FIB B I7	FIB BI7 115F	CAG AGA CAA TGA TGG ATG GTA CG	55	464bps	Designed for this study
FIB B I7	FIB BI7 115R	CCT CAG TAC TGC CAC CCT C	55		
FIB B I7	FIB BI7 248F	CCT GCA AGT TAC CAG CCA AA	55	407bps	
FIB B I7	FIB BI7 248R	TGA AAG CAG AGC ACA CAG TT	55		
FIB B I7	FIB BI7 479F	ACA CTG TCT TGC TTG AGT AGG	55	500bps	
FIB B I7	FIB BI7 479R	CTA GAC CTG CCC CAG TAC TG	55		
*PCR parameters contain an initial 10 cycles with the Ta: 55°C followed by an ac 35 cycles at Ta:50°C as described in the methods section.					ditional

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Appendix S2. Phylogenetic tree built using *BEAST.

There are variety of approaches to building a phylogenetic tree when the data consists of mitochondrial and nuclear genes. The approach we have used in the manuscript is to concatenate genes in a single matrix and estimate a tree based on the combined evidence in addition to analysing them separately (Leyva-Valencia *et al.* 2015; Patterson *et al.* 2011; Wang *et al.* 2020; Zhao *et al.* 2020). Another approach is to use a phylogenetic method that can infer the species tree while allowing discordant gene trees (Maddison & Knowles, 2006). Different phylogenetic methods can produce different phylogenetic trees. We therefore re-built the phylogenetic tree also using the *BEAST approach (Heled & Drummond, 2010) implemented in BEAST v2.6.6 (Bouckaert *et al.* 2019) in order to check the consistency of the relationships observed in the tree presented in the manuscript. For all gene sequences the HKY substitution model was selected. Tree models were linked across all mitochondrial genes. The species tree was built assuming a strict clock and a 'linear with constant root' multi-species coalescent model. The following priors were specified: the Yule tree model with a log-normal prior for birth rate, exponential priors for the clock rate and log-normal priors for the HKY transition-transversion parameters. MCMC was run for 5,000,000 iterations thinning by saving every 5,000 steps (Barido-Sottani *et al.* 2018). The species tree built using *BEAST (Fig. S.1) supported the tree obtained using the concatenated data presented in the manuscript (Figure 1) as it provided the same topology.

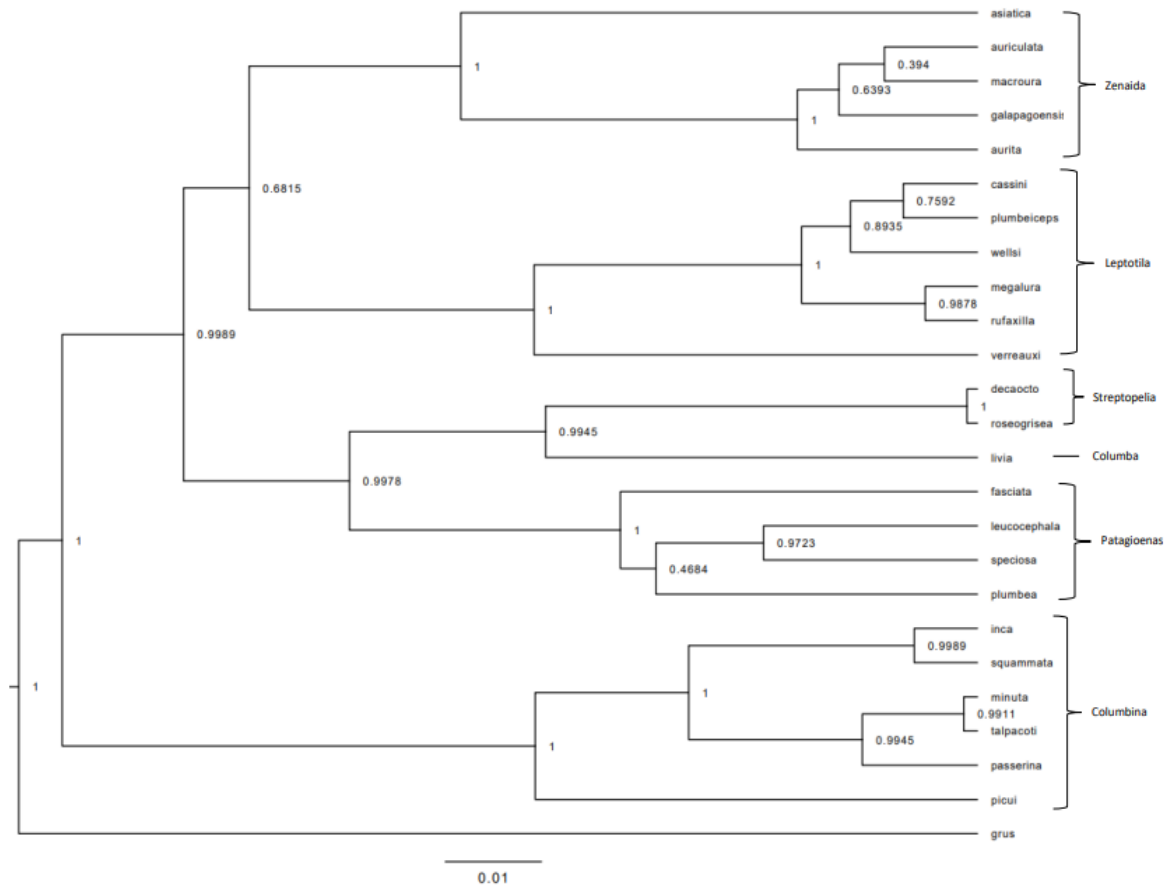

Fig. S.2 Species tree for 24 Neotropical columbid species and outgroup using the *BEAST method. Branch labels indicate the posterior probability of each node.

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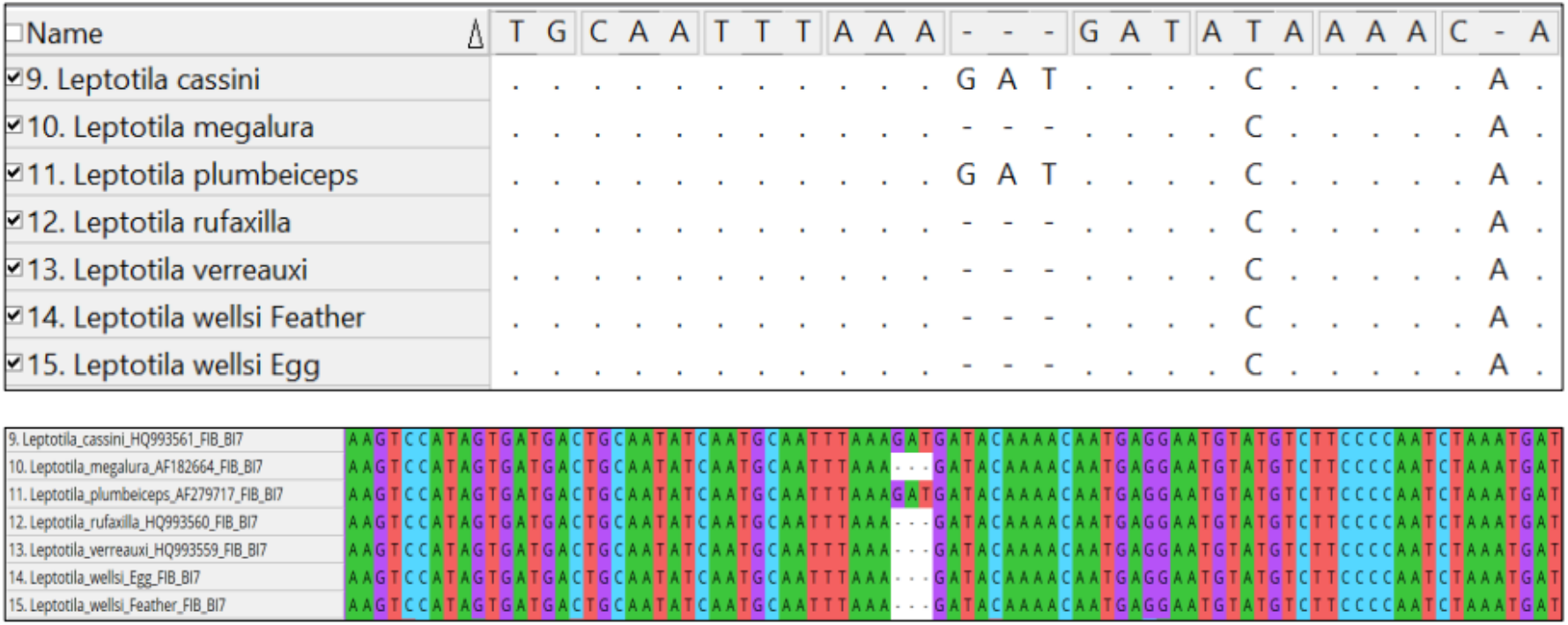
Appendix S4. Alignment of the nuclear β-fibrinogen gene for *Leptotila* species showing the indel present in *Leptotila plumbeiceps* and *Leptotila cassinii* sequences. Conserved regions are denoted by • and variable regions denoted by -. Alignments of β-fibrinogen intron 7 sequences were constructed using the method outlined in Prychitko and Moore 2003.



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Appendix S5. Phylogenetic signal lambda (λ) value per life history trait.

Life History Trait	*X* value
litter or clutch size n	1.03537452
litters or clutches_per y	7.61E-05
incubation days	0.05452824
fledging age days	6.84E-05
adult body mass g	1.0246996
female body mass g	1.02162865
male body mass g	1.02560503
no sex body mass g	1.00869828
Generation length years	1.03537452
male maturity d	4.84E-05
female maturity d	0.32784191
longevity	6.98E-05
birth or hatching weight g	7.80E-05

Appendix VIII: Gel (2% w/v agarose) image showing species specific primer optimisation displaying successful amplification of target species with corresponding sample information shown in Table 1 (below).

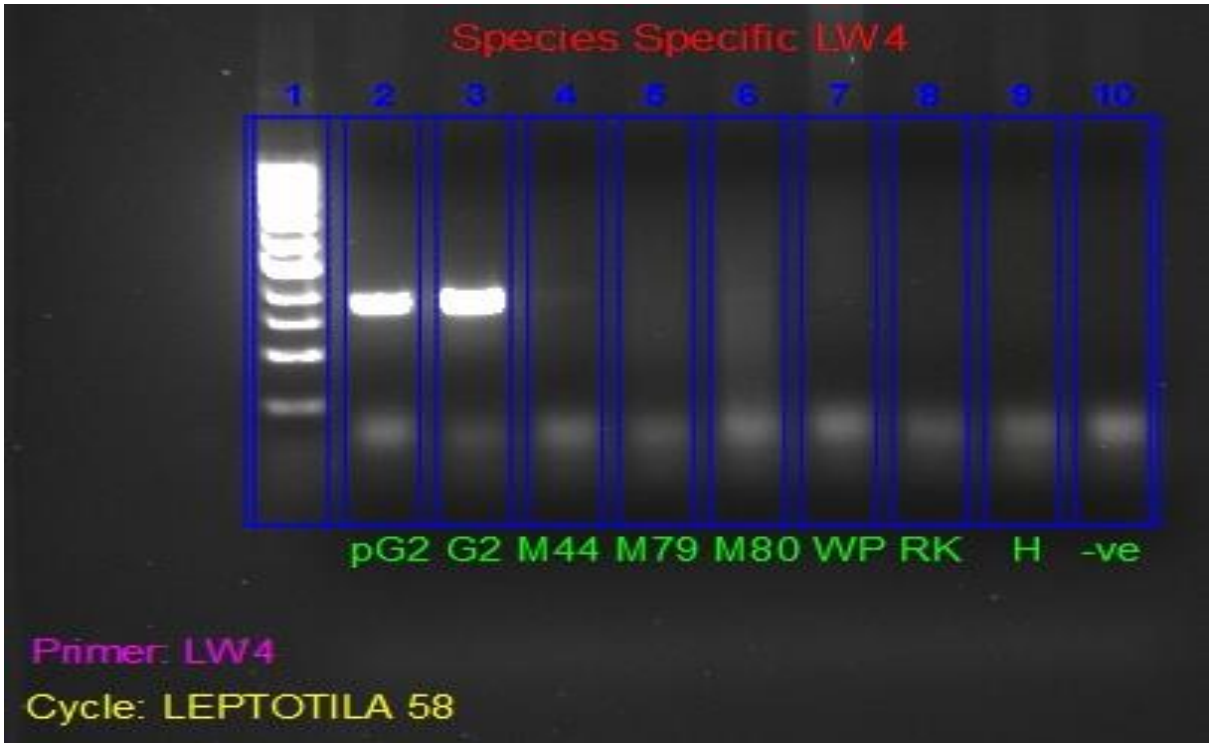


Table 1: Species specific primer optimisation sample information for corresponding gel image above.

Lane	Sample Source	Species
2	Forestry and National Parks Department of Grenada	*Leptotila wellsi*
3	Forestry and National Parks Department of Grenada	*Leptotila wellsi*
4	Forestry and National Parks Department of Grenada	*Zenaida aurita*[1]*
5	Forestry and National Parks Department of Grenada	*Patagioenas leucocephala*[2]*
6	Forestry and National Parks Department of Grenada	*Petrophassa rufipennis *[3]*
7	University of Chester	*Columba palumbus*
8	University of Chester	*Milvus milvus*
9	University of Chester	*Homo sapiens*

*NCBI's Basic Local Alignment Search Tool (BLAST®) used to predict species identification from non-invasively collected samples

[1] *Zenaida aurita* - Genbank: accession number AF182704.1; total score 1681; Per. Ident 98.24%

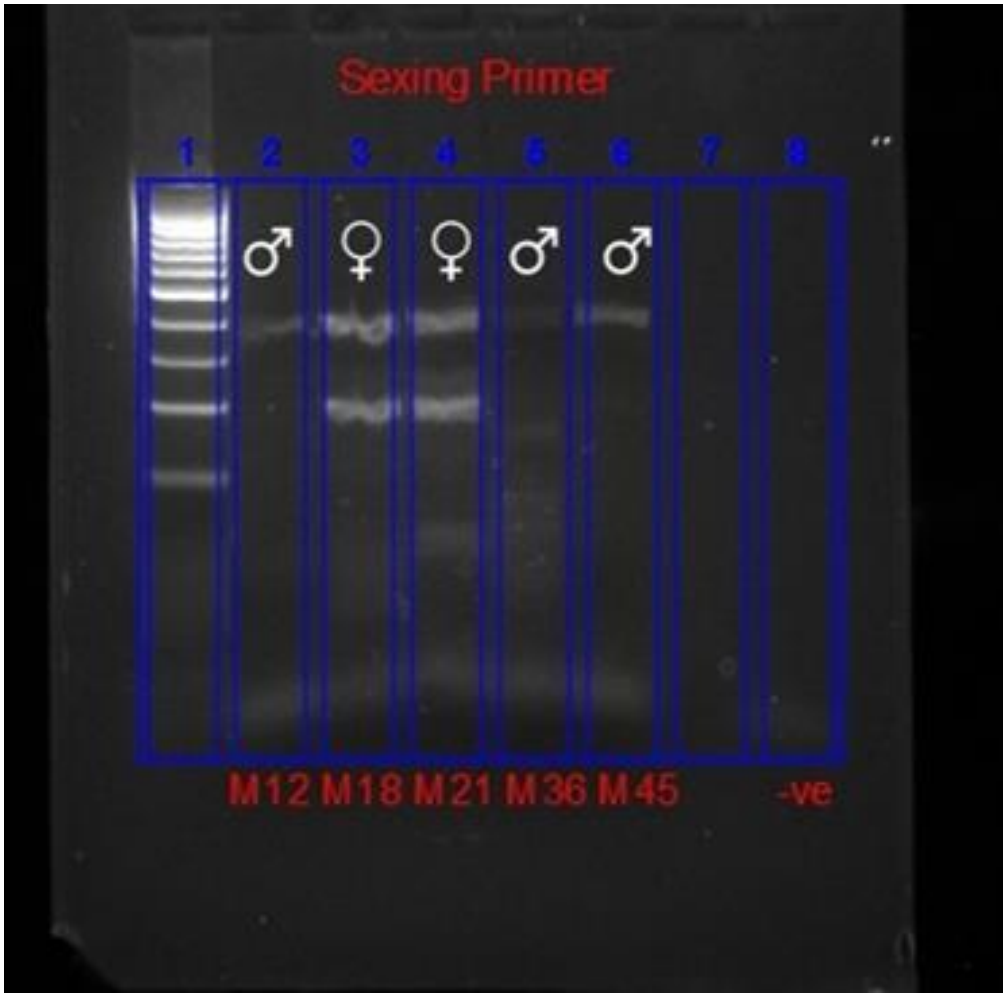
[2] *Patagioenas leucocephala* - Genbank: accession number JQ175689.1; total score 989; Per. Ident 96.77%

[3] *Petrophassa rufipennis* - Genbank: accession number KU194386.1; total score 1203; Per. Ident 92.19%

Appendix VIIII: Sample information including sample type, location, collection date, sex, NCBI BLAST scores, sequence length and sequence quality.

Sample Information							NCBI BLAST SCORES				Sequence length		Eurofins Electropheregram report Sequence quality†	
							Cyt_b_32-316*		ND2_L5126_H5766^					
Sample_name	Sample_type	Collection_Site	Collection_date	GPS_Cordinate	Sex	Haplotype	total score	Per. Ident	total score	Per. Ident	Cyt b	ND2	Cyt b	ND2
ES2	Egg shell	Mount Hartman	May-18	NA	F	A	See Chapter 3				786	965	High	High
ES3	Egg shell	Mount Hartman	09/05/2017	20P 0636525 1328392	F	A	990	96.35%	894	96.17%	786	965	High	High
F5	Body Feather	Mount Hartman	31/07/2014	20P 636381.32 1328478.4	?	A	869	95.73%	835	95.93%	786	688	High	Low
M001	Body Feather	Mount Hartman	05/07/2015	20P 0636518 1328292	M	A	970	95.56%	881	96.12%	786	965	High	High
M005	Body Feather	Mount Hartman	05/07/2015	20P 0636400 1328395	M	A	983	96.18%	872	96.58%	786	965	High	High
M010	Body Feather	Mount Hartman	06/07/2015	20P 0636398 1328374	M	A	983	96.18%	843	95.80%	786	965	High	High
M012	Body Feather	Mount Hartman	07/07/2015	20P 0636340 1328396	M	A	987	96.05%	887	95.83%	786	965	High	High
M018	Body Feather	Mount Hartman	10/07/2015	20P 636348.09 1328386.8	F	A	992	96.07%	881	96.28%	786	965	High	High
M021	Body Feather	Mount Hartman	10/07/2015	20P 636348.63 1328269.2	F	A	981	96.32%	869	96.06%	786	965	High	High
M036	Body Feather	Mount Hartman	17/05/2017	20P 0636329 1328024	M	A	983	96.47%	865	96.05%	786	965	High	High
M045	Body Feather	Mount Hartman	17/05/2017	20P 0636334 1327817	M	NA	976	96.15%	872	95.60%	786	688	Low	High
M055	Body Feather	Mount Hartman	17/05/2017	20P 636362.23 1328294.0	M	A	979	96.02%	874	96.76%	786	965	High	High
M064	Body Feather	Mount Hartman	09/05/2018	20P 0636396 1328369	M	A	974	96.14%	874	96.76%	786	965	High	High
M107	Body Feather	Mount Hartman	NA	20P 636342.12 1328310.5	M	A	950	96.06%	841	95.95%	786	965	High	High
M108	Body Feather	Mount Hartman	NA	20P 636362.21 1328297.7	F	A	972	95.70%	839	95.95%	786	965	High	High
M110	Body Feather	Mount Hartman	NA	20P 636498.41 1328276.2	?	NA	983	96.18%	850	95.33%	786	688	High	Low
M111	Body Feather	Mount Hartman	NA	20P 636342.22 1328288.4	M	A	987	96.05%	889	95.98%	786	965	High	High
M116	Body Feather	Mount Hartman	NA	20P 636425.49 1328342.3	M	A	992	96.07%	880	96.28%	786	965	High	High
G11	Secondary Feathers	Perseverance	May-17	NA	M	A	See Chapter 3				786	965	High	High
P05	Body Feather	Perseverance	07/07/2015	20 P 0636199 1338500	?	NA	994	95.93%	867	96.39%	786	688	High	Low
P06	Body Feather	Perseverance	07/07/2015	20 P 0636193 1338495	?	A	983	96.04%	837	96.27%	786	965	High	High
P22	Body Feather	Perseverance	18/05/2017	20P0636197 UTM1338407	M	B	972	95.99%	843	96.12%	786	965	High	High
P28	Body Feather	Perseverance	18/05/2017	20P0636207UTM1338403	M	B	979	95.88%	874	96.42%	786	965	High	High
P31	Body Feather	Perseverance	18/05/2017	20P0636288 UTM1338453	M	B	1003	96.25%	872	95.61%	786	965	High	High
P32	Body Feather	Perseverance	18/05/2017	20P0636288 UTM1338453	M	B	974	96.29%	876	95.93%	786	965	High	High
P34	Body Feather	Perseverance	18/05/2017	20P0636170 UTM1338434	M	B	983	96.18%	843	96.13%	786	965	High	High
P39	Body Feather	Perseverance	18/05/2017	20P0636170 UTM1338434	M	NA	985	96.33%	850	96.32%	786	688	High	Low
P40	Body Feather	Perseverance	18/05/2017	20P0636170 UTM1338434	?	B	1002	96.39%	876	96.25%	786	965	High	High
P45	Body Feather	Perseverance	18/05/2017	20P0636288 UTM1338456	?	NA	996	96.22%	835	96.27%	786	688	High	Low
P46	Body Feather	Perseverance	18/05/2017	20P0636288 UTM1338456	?	NA	1007	96.41%	822	94.56%	786	688	High	Low
*Top 14 search results were Leptotila species; Total score and Per. Ident values given for top match: Leptotila cassini Accession Number: HQ993505.1														
^Top 28 search results were Leptotila species; Total score and Per. Ident values given for top match: Leptotila plumbeiceps Accession Number: HQ993544.1														
† Sequence quality based on average quality score reported on electropheregram per sequence where high is defined at average quality >=30 and high peak calls and low defined as average quality is 0-29 and low peaks calls.														

Appendix X: Example gel (3% w/v agarose) image showing the successful amplification using universal avian sexing primers P8 and P2 (Griffiths *et al.* 1998) with one band at the 400bp position for males and two bands at the 400bp and 200bp position for females.



Appendix XI: Sensitivity Analysis

Sensitivity analysis (Figure 1 -below) for percentage of females breeding showed that the probability of extinction did not vary substantially across the simulated parameter values. Growth rates for reproductive rates remained negative even at -50% females breeding. Variation in the percentage of juvenile and adult mortality rates showed that the probability of extinction did not vary substantially across the simulated parameter values. Growth rates for mortality rates remained negative even at -50% mortality of juveniles and adults. The frequency and severity of both hurricanes and habitat loss did not vary the probability of extinction or population growth rate. For all simulated parameters the mean size of the extant population was not considerably different to baselines values. Sensitivity analysis showed that change of mortality rates of juvenile and adult birds, reproductive parameters, hurricane and habitat loss frequency and severity did not change population trajectories.

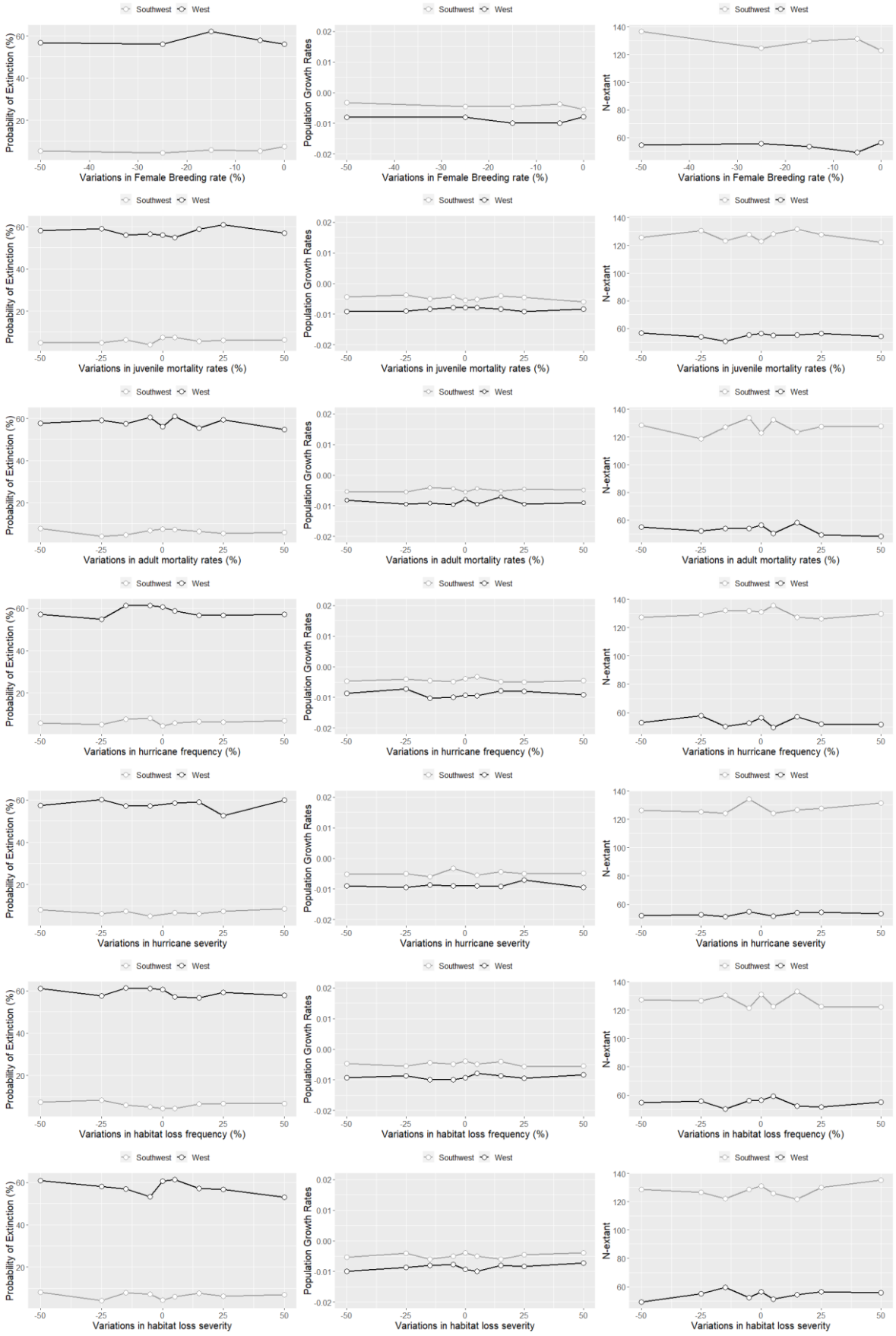


Figure 1. Sensitivity analysis of key demographic parameters (number of females breeding, juvenile and adult mortality rates, hurricane and habitat loss frequency and severity) on probability of extinction, population growth rates and mean size of the extant population (N-extant) for the two populations of Grenada Dove. Points represent tested parameter values. Baseline values used in the standard model are represented by 0.

Appendix XII: Scripts and Data

All files listed in this appendix are available at: Appendix XII: Scripts and Data

Data

- Grenada Dove Sequence data
- Life History Trait data
- Systematic Review data
- Vortex input files

Software

- **MEGA X**
  - https://www.megasoftware.net/
- **Sequencer 5.4.6**
  - http://genecodes.com/
- **R and R studio**
  - https://www.r-project.org/
  - https://www.rstudio.com/
- **Vortex 10**
  - https://scti.tools/vortex/

Scripts

- **Chapter 2**
  - Systematic review key word generator
    - Litsearchr R Package https://elizagrames.github.io/litsearchr/#
  - Code to plot results
- **Chapter 3**
  - Statistical analysis
- **Chapter 4**
  - Phylogenetic Comparative Analysis
    - RPhylopars R package https://besjournals.onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2F2041-210X.12612&file=mee312612-sup-0003-AppendixS3.pdf
  - Model Validation
  - Phylogenetic signal
  - Code to plot results
- **Chapter 5**
  - Population genetic analysis
    - PopGenome R Package https://rdrr.io/cran/PopGenome/f/inst/doc/An_introduction_to_the_PopGenome_package.pdf
  - Code to plot results
- **Chapter 6**
  - ANOVA
  - Code to plot results