Conservation genetics of granivorous birds in a heterogeneous landscape: the case of the Black-throated Finch (*Poephila cincta*)

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I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis	**C. Smith-Keune** Signature: **M.G. Gardner** Signature: **D.B. Hardesty** Signature:

This thesis is dedicated to my family and you as a reader.

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ABSTRACT

More than a third of granivorous birds have declined in Australia as a result of substantial landscape changes through grazing, altered fire regimes and land clearing for agricultural purposes. Understanding the genetic impact of the granivore decline is important to determine appropriate conservation management strategies because declining populations are vulnerable to negative genetic processes such as inbreeding depression, genetic bottlenecks and genetic drift, all of which could lead to decreased genetic diversity and reduced evolutionary potential.

This thesis uses the threatened Black-throated Finch as a case study to investigate the importance of applying genetic information in the conservation of declining granivorous birds in Australia. Specifically, I developed 18 novel microsatellite markers using next generation sequencing technology for the Black-throated Finch and tested these for cross-species amplification success in two other co-occurring grass finch species, the Double-barred Finch (*Taeniopygia bichenovii*) and the Chestnut-breasted Mannikin (*Lonchura castaneothorax*) (Chapter III). Using these microsatellite markers, I then examined the genetic diversity and population structure of four major surviving populations of the Black-throated Finch in northern Queensland, Australia (Chapter IV). Fine-scale spatial genetic structuring was also analysed among individuals around a man-made dam in northern Queensland to determine the relationship between local heterogeneous landscapes that birds occupy and the genetic distance between individuals within tens of square kilometres (Chapter V). Last, I evaluated conservation values of the Black-throated Finch in captivity by comparing the genetic diversity, effective population sizes, inbreeding and relatedness between *in situ* and *ex situ* populations (Chapter VI).

The sampled four, widely-dispersed wild populations of the Black-throated Finch had similar levels of genetic diversity. There was a significant separation between northern and southern subspecies based on genetic structuring analysis (between subspecies F_{CT} = 0.034, $p < 0.001$). At the local scale, individual birds demonstrated strong spatial genetic structuring separated by the Ross River Dam in the Townsville region. Environmental Niche Modelling identified local landscape features such as vegetation structure and the presence of water as having a strong association with the occurrence of the Black-throated Finch. The individual genetic distance was weakly, but significantly correlated with the geographical distance (Mantel $R = 0.081$, $p < 0.001$) and the landscape resistance distance (Mantel $R = 0.083$, $p < 0.001$). Samples from captive birds showed significant differentiation from wild birds. Birds in captivity had significantly lower levels of genetic diversity (average $H_O = 0.35$ among captive populations versus average $H_O = 0.45$ in the wild; average $r = 2.34$ in captivity versus $r = 3.08$ in the wild); smaller effective population sizes; higher levels of inbreeding ($F = 0.114$ in the wild versus $F = 0.216$ in captivity) and more admixed genetic structuring compared with their wild counterparts.

Results of my thesis have provided evidence that the northern and southern subspecies of the Black-throated Finch are genetically differentiated, but the differentiation is weak. The similar level of genetic diversity across all wild populations suggested that the genetic exchange was historically high among sampled populations despite demonstrated low levels of demographic connectivity and recent population declines. Fine-scale genetic analysis of birds around the Ross River Dam from the Townsville population demonstrated that current landscape features (an open lake in particular) and spatial separation had a combined effect on the genetic structure of the local Black-throated Finch population. In addition, birds in captivity have lost genetic variability to

some degree and increased levels of inbreeding potentially may reduce the viability. However, birds descended from extinct wild populations are still maintained in captivity, providing potential genetic sources for future captive breeding programmes.

Conservation management strategies should therefore 1) prioritise *in situ* approaches, such as frequent monitoring of population trends, identifying and maintaining existing suitable habitats, increasing the connectivity between suitable habitats by minimising dispersal barriers (large open waters, dense invasive plants and heavily grazed landscapes); and 2) consider the conservation values of captive birds (particularly birds with known origins) in establishing possible *in situ* breeding stocks if *ex situ* conservation strategies fail to increase the population viability.

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LIST OF ABBREVIATIONS

(In alphabetic order)

ABBBS	Australian Bird and Bat Banding Scheme
AFLP	Amplified Fragment Length Polymorphism
AGRF	Australian Genomic Research Facility
AMOVA	Analysis of Molecular Variance
AUC	Averaged Area Under the Curve
BDT	BigDye® Terminator
BLAST	Basic Local Alignment Search Tool
DEM	Digital Elevation Model
ENM	Ecological Niche Modelling
GCDI	Ground Cover Disturbance Index
GIS	Geographic Information System
GOFI	Gouldian Finch
HWE	Hardy Weinberg Equilibrium
IAM	Infinite Allele Model
IBD	Isolation by Distance
IUCN	International Union for Conservation of Nature
LCP	Least-cost Path
LD	Linkage Disequilibrium
MAI	Maximum Avoidance of Inbreeding

MCMC	Markov Chain Monte Carlo
NDII	Normalised Difference Infrared Index
NDVI	Normalised Difference Vegetation Index
NDWI	Normalised Difference Water Index
OR	Omission Rate
PCR	Polymerase Chain Reaction
PIC	Polymorphic Information Content
RAPD	Random Amplified Polymorphic DNA
RE	Regional Ecosystem
RFLP	Restriction Fragment Length Polymorphism
ROC	Receiver Operator Characteristic
SLATS	Statewide Landcover and Tree Study (Queensland, Australia)
SMM	Single Mutation Model
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
STR	Short Tandem Repeat
TPM	Two Phase Model
TWI	Topographic Wetness Index
USGS	United States Geological Survey

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CHAPTER I

Background and general introduction

(Photo credit: L. Stanley Tang)

BACKGROUND

Granivorous birds in decline

Granivorous (seed-eating) birds are a major component of the avifauna in most terrestrial communities. In Australia, 20% of the land bird species are granivores (Franklin et al. 2000). These birds have evolved independently within various taxa (finches, parrots, quail-like birds, pigeons and doves), and they are characterised by an array of specific morphological and ecological features, such as a beak that is suitable for extracting seeds, an ability to form large feeding aggregations, and a requirement for frequent water consumption and seed resources (Wiens and Johnson 1977). The specific resource requirements have made granivorous birds particularly vulnerable to changes in the landscapes they occupy. Approximately one-third of Australian granivorous bird species have declined and they represent more than a quarter of all threatened land bird taxa in Australia (Figure I-1) (Garnett and Crowley 2000a).

The decline of granivorous birds has occurred unevenly across the country. The decline is greatest in northern Australia, where the tropical and subtropical savannahs hold the largest granivorous bird assemblages (State of the Environment Advisory Council 1996, Franklin et al. 2005). Declines have also occurred in the arid zone (Reid and Fleming 1992). For example, six finch species are under threat and they all occur predominantly in northern Australia. These finches include the Gouldian Finch (*Erythrura gouldiae*, Endangered), the Pictorella Mannikin (*Heteromunia pectoralis*, Near Threatened), the White-bellied Crimson Finch (*Neochmia phaeton evangelinae*, Endangered), the Star Finch (*Neochmia ruficauda clarescens*, Endangered; *N. r. ruficauda*, Extinct; *N. r. subclarescens*, Near Threatened), the Southern Black-throated Finch (*Poephila cincta cincta*, Vulnerable) and the Diamond Firetail (*Stagonopleura guttata*, Near Threatened).

Six parrots, four quail-like birds, and five pigeons and doves from northern Australia are also under threat (Garnett and Crowley 2000a).

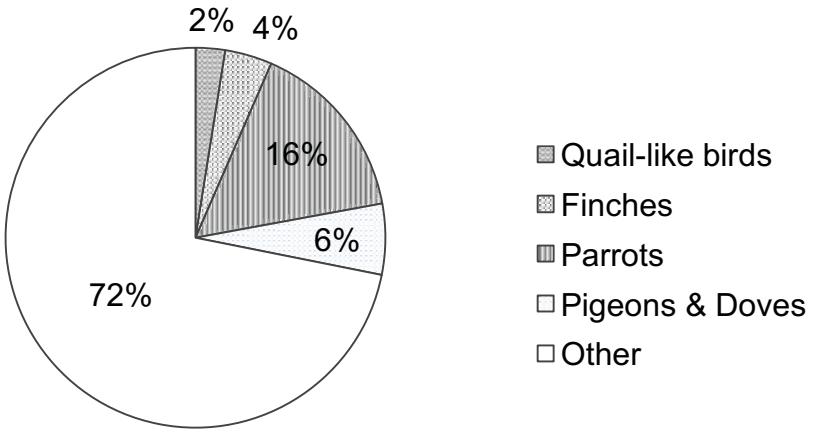

Figure I-1 Percentage contributions of different bird groups to the threatened avifauna of Australia. Data are from the Action Plan for Australian Birds (Garnett and Crowley 2000a)

There have been more substantial landscape changes in southern Australia than in the north since European settlement. Nonetheless, more recent changes in northern Australia have resulted in the range decline of granivore species (Woinarski 1999). The decline is likely to be more severe in areas where grazing intensity is higher and pastoralism has been established longer in northern Australia (Whitehead 2000, Sharp and Whittaker 2003). In particular, extensive grazing alters grass species composition, productivity, seed production, and therefore overall food resources for granivores (e.g. Walker et al. 1997). Grazing in combination with prolonged drought is also likely to be a major factor in the decline of the Star Finch (*N. r. ruficauda*). This is because the Star Finch usually occurs in long grass close to water and, during droughts, the continuous presence of livestock around these water bodies severely alters this critical habitat (Holmes 1998).

The decline may be related to the changes in frequency and timing of fire since European settlement (Walker et al. 1997, Russell-Smith et al. 2003). The general trend is of decreased frequency on pastoral properties and increased frequency in high-rainfall, non-pastoral areas (Williams et al. 2002, Andersen et al. 2005). These changes have important implications for biodiversity. The experimental fire regimes at Kakadu National Park have had a combination of positive and negative impacts on only five out of 25 bird species. Some birds including the Brown Goshawk (*Accipiter fasciatus*) and the Red-backed Kingfisher (*Todiramphus pyrrhopygia*) showed positive responses (Andersen et al. 2005). However, the impact of changes in fire regimes on granivorous birds is generally negative. The habitat of the Golden-shouldered Parrot (*Psephotus chrysopterygius*) has also been altered due to changes in the fire regime creating a coarser fire mosaic limiting the dispersal of the bird during the wet season (Garnett and Crowley 2002). Increased frequency or extent of fires also results in increased mortality of eggs and hatchlings of the Partridge Pigeon (*Geophaps smithii*) in the dry season as the bird nests on the ground within clumps of grass (Woinarski 2004).

The loss of granivorous birds is also related to changes in habitat arrangement and vegetation structure of the landscape. Heterogeneous landscapes, with their greater diversity of habitats, provide opportunities for granivores under different environmental conditions (Woinarski 1999, Franklin et al. 2005). Grazing, invasive species and fire all change the landscape, particularly the understorey vegetation. Granivorous birds in savannahs are susceptible to such environmental variation because of their specialised diet (Franklin et al. 2005). For example, the Gouldian Finch has a restricted diet in the wet season with its food limited to cockatoo grass (*Alloteropsis semialata*) and golden beard grass (*Chrysopogon fallax*). Both of these grasses are selectively grazed by cattle and horses leading to a reduction of critical wet season seed resources for the bird

(O'Malley 2006). Invasive/exotic plant species are also involved in landscape changes. The rapid spread of exotic pasture grasses and woody weeds into savannahs can replace native species reducing available food resources for granivores, and increasing the fuel load leading to increased fire intensities (Rossiter et al. 2003). The introduced Rubber Vine (*Cryptostegia grandifolia*), for example, has invaded and replaced the native vegetation in some areas of the habitat of the White-bellied Crimson Finch (*Neochmia phaeton evangelinae*) possibly contributing to the decline of this bird (Garnett and Crowley 2000a).

Genetic consequences of the decline in granivorous birds

The decline of granivorous birds may have negative genetic consequences, such as inbreeding depression, loss of neutral genetic variation, fixation of deleterious alleles and genetic bottlenecks, which are usually associated with small or fragmented populations (Herdrick 2011). Population fragmentation and isolation may have detrimental effects on the fitness and viability of surviving populations (Ferrière et al. 2004). Failure to consider genetic consequences of the decline of a species may result in underestimation of the probability of extinction and the implementation of inappropriate conservation strategies (Frankham and Ralls 1998, Frankham 2005). For example, a molecular study of the Gouldian Finch identified recent population declines followed by episodes of population expansion throughout northern Australia. Although no genetic evidence of inbreeding or recent bottlenecks was found in wild populations of the Gouldian Finch, heterozygosity was significantly lower. There was also evidence for a recent population bottleneck in a captive stock which was part of a reintroduction programme for the maintenance and persistence of the species (Esparza Salas 2008).

Introducing additional individuals from diverse wild populations into this captive population may increase the genetic variability of released birds (Esparza Salas 2008).

Genetic data can also identify distinct populations, guiding management actions. For example, using microsatellite DNA markers, the Yellow Rail (*Coturnicops noveboracencis*) of Oregon, USA was identified as a genetically distant population with the lowest genetic diversity comparing with other major populations of the species (Miller et al. 2012). The Black Grouse (*Tetrao tetrix*) in Great Britain, on the other hand, has become fragmented and isolated into three genetically distinct populations with little migration between them (Höglund et al. 2011). The genetic differentiation observed is likely due to genetic drift in small and fragmented sub-populations and, because these events are recent, there may be little advantage to managing these populations separately (Höglund et al. 2011).

Landscape features and heterogeneity are often reflected in population genetic structure within and among species (Miller and Haig 2010, Safner et al. 2011). Changes in landscapes are often associated with habitat loss, which contributes to population decline possibly resulting in reduced genetic diversity of remaining populations (Templeton et al. 1990, Keyghobadi 2007). Habitat loss and fragmentation due to landscape changes lead to population bottlenecks and local extinction, which in turn result in the loss of overall genetic diversity (Keyghobadi 2007). Measures of genetic differentiation, for example, are negatively associated with percentage of forest cover between breeding sites of the Golden-cheeked Warbler (*Dendroica chrysoparia*) in Texas, USA, and positively associated with percentage of agricultural land between sites (Lindsay et al. 2008). Although the Golden-cheeked Warbler is a migratory species, the vast majority of individuals nest within 4km of where they were born. The

observed high level of genetic differentiation among populations was due to the agriculture related fragmentation of its forested breeding habitat (Lindsay et al. 2008). Other landscape features are also associated with the genetic structure of birds. For example, water barriers reduce gene flow between populations of the Song Sparrow (*Melospiza melodia*) and generate geographic variations in the genetic structure (Wilson et al. 2011).

STUDY SPECIES

The Black-throated Finch is endemic to Australia and was once distributed from north-eastern New South Wales to Cape York Peninsula in grassy woodlands dominated by eucalypts, melaleucas or acacias (Baldwin 1976). It typically occurs near water sources, where it feeds on grass seeds and may also hawk after flying insects (Zann 1976) (Rechetelo unpublished data and pers. comm.). There are two subspecies of *P. cincta* (Figure I-2). The northern (black-rumped) form *P. c. atropygialis* occurs north from Mareeba on the Atherton Tablelands, Queensland and across Cape York Peninsula, and its populations are widespread and secure. The southern (white-rumped) form *P. c. cincta* once ranged from the Atherton Tablelands to as far south as north-east New South Wales, but it has disappeared from much of the southern extent of its range (Schodde and Mason 1999) (Figure I-3).

Since 1998, the southern form has been recorded in only four bioregions from central to north Queensland and some evidence suggests that the species may have declined by 50% in the past 10 years (Environment Australia 2000, Black-throated Finch Recovery Team 2007). Currently, this subspecies is listed as “Endangered” under the national legislation (*Environment Protection and Biodiversity Conservation Act* 1999) and under

the Queensland state legislation (*Nature Conservation Act* 1992). The Black-throated Finch is listed as "Presumed Extinct" in New South Wales under the state legislation (*Threatened Species Conservation Act* 1995). Under international conventions, at the species level, it is listed as "Near Threatened" in the IUCN Red List of Threatened Species 2011 and under Appendix II of the CITES 2011.

Figure I-2 The Black-throated Finch (*Poephila cincta*), northern subspecies (*P. c. atropygialis*) with a black rump (left) and southern subspecies (*P. c. cincta*) with a white rump (right). Photos by L.S. Tang

The decline of the Black-throated Finch began early in the 1900s with the expansion of pastoralism into its habitat (Franklin 1999). Recent sighting records indicate that the distribution of *P.c. cincta* is severely fragmented. To date, there has been no reliable information or accurate estimate of the occurrence, size, number or the natural fluctuations of the populations and subpopulations of the species. However, the decline of *P. c. cincta* is continuing according to the on-going monitoring activities conducted by the Black-throated Finch Recovery Team (Black-throated Finch Recovery Team 2007). Possible threats to the bird include: (1) clearance and fragmentation of woodlands, riparian habitats and *Acacia* scrublands; (2) degradation of habitat by domestic livestock and rabbits, including the alteration of vegetation structure, the availability of food during the wet season and the reduction of fuel loads, (3) invasion

of habitat by exotic plants, including grasses; (4) trapping for aviculture; and (5) predation by introduced animals, such as feral cats (Natural Resource Assessment Environmental Consultants 2005, Black-throated Finch Recovery Team 2007).

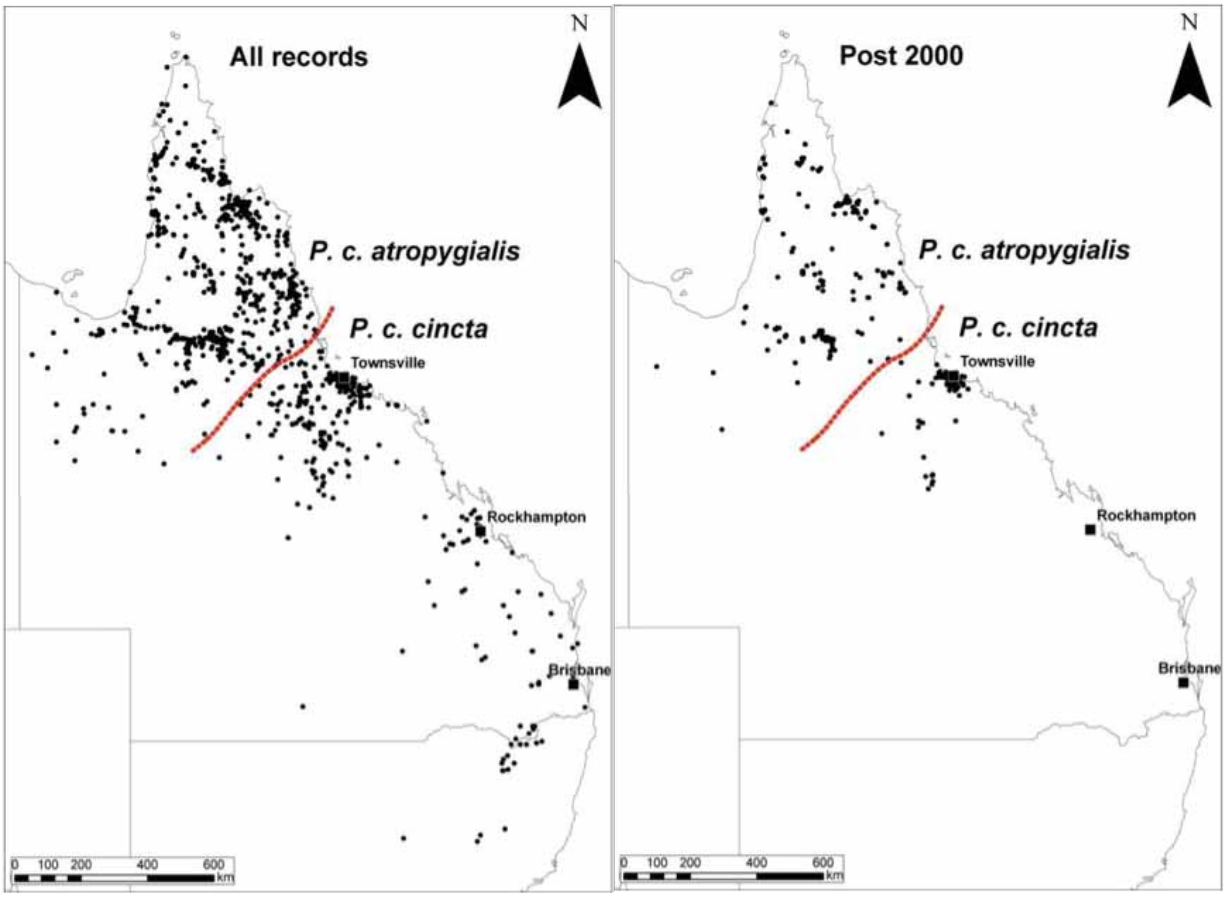

Figure I-3 Distribution maps of the Black-throated Finch (*Poephila cincta*) (from the Black-throated Finch Recovery Team). The dotted line indicates an estimated boundary between northern (*P. c. atropygialis*) and southern subspecies (*P. c. cincta*). Each dot indicates a recognised and recorded sighting. (a) all historical recorded sightings and (b) sightings recorded after 2000.

The movement patterns of the Black-throated Finch are poorly known. The southern subspecies was considered as resident in its known locations including northern New South Wales, northern and central Queensland in Australia (Longmore 1978, Morris et al. 1981, Britton and Britton 2000, Forshaw et al. 2012). Recent sighting records and research has shown that the Black-throated Finch in the Townsville region can move up to 20km (J. Rechetelo, unpublished data). Some movements may be undertaken due to

changes in food availability as a result of increased rainfall or drought (Baldwin 1976, Mitchell 1996). Post-breeding dispersal appears to be limited as birds are still encountered at their breed sites during non-breeding seasons (Mitchell 1996, Natural Resource Assessment Environmental Consultants 2007).

Given the limited movement of the Black-throated Finch (particularly the southern subspecies) and its fragmented populations, it is likely the risk of extinction of the bird is high. In addition, rapid changes in the heterogeneous landscape that the bird uses could mean that sub-populations become fragmented leading to an increased probability of deleterious genetic consequences.

GENETIC APPROACHES

Using molecular data to examine genetic variation in order to understand the ecological and evolutionary processes within and among population and/or species has become a great complementary tool to traditional methods using phenotypic (e.g. morphology, physiology and behaviour) and ecological data (Avise 2004, Sunnucks and Taylor 2008). Molecular information allows us to directly quantify genetic diversity, measure population admixture, estimate inbreeding, trace historical patterns of dispersal and to identify taxonomic lineages (Avise 2004). With the rapid advancement of molecular technology and development of more robust molecular markers, an increasing variety of approaches have become available, providing an array of powerful tools in the field of ecological research.

Marker choice

Molecular markers allow the relatively rapid detection and characterisation of genetic variation. However, no single molecular marker is ideally suited to all evolutionary and ecological studies. Therefore, the choice of molecular markers is crucial in order to address particular research questions. For example, some markers such as microsatellite DNA and DNA sequences that host single nucleotide polymorphisms (SNPs) exhibit higher mutation rates and evolve fast enough to infer recent evolutionary histories. Other markers, such as the genomic DNA sequence, provide better coverage of the entire genome. Mitochondrial DNA, on the other hand, is only maternally inherited, and is thus useful for detecting female biased genetic structure (Balkenhol et al. 2009, Wang 2011). For population and individual level studies, the molecular markers need to be fast evolving and highly variable (Herdrick 2011). The following molecular markers are commonly used in population studies.

Microsatellite DNA/Single Sequence Repeats (SSRs)

Microsatellite loci have been the most widely used molecular markers in conservation genetic studies. They are DNA sequences composed of short tandem repeats (STRs) or simple sequence repeats (SSRs) of two to six nucleotides. For example, $(AC)_{10}$ is a sequence of 10 repeated units of the dinucleotides AC, i.e. ACACACACACACACACACAC. These loci are generally codominant, selectively neutral and multi allelic. The number of repeats is highly heterogeneous. Unlike mitochondrial DNA, microsatellites often present high levels of inter- and intra-specific polymorphism, which can be easily determined by electrophoresis or automated size separation of the polymerase chain reaction (PCR) amplified DNA fragments. Therefore, microsatellite markers are widely used in gene mapping and population

genetic studies (review in Goldstein and Pollock 1997, McDonald and Potts 1997). They can also be used to evaluate allele relatedness (Goldstein et al. 1995) .

Microsatellite loci are the first choice in most conservation genetic studies, but they have some drawbacks. Primers amplifying microsatellites have to be specifically designed for each species. The transferability of these primers decreases significantly as species become less closely related (Jarne and Lagoda 1996). Therefore, the development of microsatellite primers is potentially time consuming and expensive. Moreover, in the genome of some species, microsatellites can be sparse and so difficult to find (Zane et al. 2002, Schlötterer 2004). As a result, the use of microsatellites in less studied species with little genetic information (non-model species) is limited because of insufficient marker resources (Luikart et al. 2003). However, with improvements in sequencing technologies and the greater availability of sequence databases, the discovery and typing of microsatellites in non-model species has become more accessible (Hudson 2008).

Single Nucleotide Polymorphisms (SNPs)

SNPs refer to the bi-allelic variations of single nucleotides within almost identical DNA sequences from individuals of the same species. Heterozygotes are identified by different nucleotides at specific sites whereas homozygotes show the same nucleotide. SNPs are by far the most abundant and accurate type of polymorphism in genomes (Schlötterer 2004). Consequently, these markers have become increasingly important in genotyping (especially large genome scanning) and biomedical research (Williams et al. 2010). They are useful markers for studies of disease development (e.g. Johnson et al. 2007, Amos et al. 2008) and genetic adaptation (e.g. Mauricio et al. 2003, Namroud et al. 2008). SNPs have also been used more frequently than other markers in landscape

genetics to quantify genetic differences between individuals, populations and species (Manel et al. 2010). In vertebrates, for example, one SNP occurs on average every 100 to 1000 base pairs and is usually in linkage disequilibrium with other SNPs (Vignal et al. 2002). Unfortunately, detecting SNPs is a costly time-consuming task and generally requires *a priori* information on the studied genome (Morin et al. 2004), with the exception of newly developed technologies such as the restriction site associated DNA sequencing (RAD-seq) and genotyping by sequencing (GBS), which can be used in non-model species (Jiang et al. 2016). Also, the marker resources are not readily available for most non-model species.

Mitochondrial DNA & Restriction Fragment Length Polymorphisms (RFLPs)

Mitochondrial DNA play important roles as genetic markers in population and conservation biology. Their popularity comes from their uniparental inheritance (usually maternal), lack of recombination and simple composition of sequences (Harrison 1989). As a result, these markers have been widely used in phylogenic and phylogeographic research to trace maternal genealogies and detect dispersal events (Herdrick 2011). Mitochondrial DNA also have relatively fast rates of divergence (especially some non-coding regions) that, in principle, makes them useful for detecting variations among individuals within a landscape at temporal scales (Vandergast et al. 2007, Swart et al. 2009, Measey and Tolley 2011). However, these markers do not always provide enough variation among individuals at fine spatial scales, which has limited their applications in landscape genetics (Holderegger and Wagner 2008, Bohonak and Vandergast 2011).

Variation of these markers is usually assessed by DNA sequencing or by restriction enzymes (e.g. EcoR1). DNA sequencing is fast and accurate and it has been widely used

for mitochondrial DNA based population genetic analyses. Restriction enzymes recognise specific sites in mitochondrial DNA. The spacing between each two sites may vary in length among individuals or populations resulting in fragments of different lengths (restriction fragment length polymorphisms, RFLPs) after DNA digestion. However, this technique requires large amounts of DNA and produce only limited information, so its application has been limited to some microorganisms including pathgens (Brudey et al. 2006) and fungi (e.g. Kinloch et al. 1998).

Estimation of genetic diversity and genetic structure

Genetic diversity, estimated using either allele or genotype frequencies, is important in population studies as it determines the ability of any population to adapt and evolve to changing conditions with implications for their long-term survival. Descriptive measures, including allelic richness, allelic diversity and observed heterozygosity, can help characterise genetic diversity of each population (Beebee and Rowe 2008, Freeland et al. 2011). For example, a deficiency in observed heterozygosity could indicate that the population is not in Hardy-Weinberg Equilibrium and may be undergoing certain genetic processes, such as inbreeding or genetic drift.

Another important feature of population genetic studies is the determination of genetic structure and the measurement of levels of gene flow between populations. Gene flow affects the ecology and evolution of natural populations (Hanski and Gilpin 1997). For example, a high level of gene flow reduces divergence and so can decrease the likelihood of reproductive isolation. Frequent gene flow among populations can enhance local adaptation to heterogeneous landscapes (Lenormand 2002). Furthermore, gene flow also affects important ecological properties such as species extinction rates (Epps et al. 2005), evolution of species distributions (Alleaume-Benharira et al. 2006),

population persistence (Palstra and Ruzzante 2008) and the synchrony of population size changes (Richards et al. 1999).

Many statistical approaches have been proposed to evaluate gene flow. Most commonly, gene flow is referred to as N_em, the product of individual effective population size N_e and migration rate m, and is estimated from frequencies of genetic markers by assuming equilibrium between gene flow and neutral genetic drift (Mallet 2001). Under the "island model", where migrants can come from anywhere in the metapopulation (therefore the gene frequency is constant), gene flow can be estimated by calculating the fixation index $F_{\mathrm{ST}} = \frac{1}{1+N_em}$ (Wright 1969). According to this equation, when populations are small and/or migrations between populations become so rare that $N_em \ll 1$ drift outweighs gene flow and $F_{\mathrm{ST}} \to 1$. Conversely, if populations are large and/or migrations are frequent enough so that $N_em \gg 1$, gene flow dominates drift and $F_{\mathrm{ST}} \to 0$. In other words, the greater the F_{ST} is, the greater the genetic differentiation between populations becomes. This relationship can broadly apply in more realistic population structures, such as the "stepping stone" model, where migrants are exchanged mainly between local subpopulations (Slatkin and Barton 1989).

F_{ST} is suitable for datasets consisting of unlinked neutral markers, such as single nucleotide polymorphisms (SNPs) with two alleles per locus. F_{ST} provides a basis for measuring genetic distance when population divergence is caused by genetic drift (Reynolds et al. 1983). Depending on the properties of selected markers, there are a number of other related statistics developed as analogues of F_{ST}. For example, the microsatellite markers assume the stepwise mutation model (SMM), where novel alleles are created by adding or deleting repeated units of microsatellite sequences. Therefore, R_{ST}, an analogue of F_{ST} incorporating SMM, is thought to reflect more accurately in the

evolution of microsatellite markers (Slatkin 1995, Balloux and Lugon-Moulin 2002). Other statistics, such as the estimate of divergence D_{est}, derived from the true allelic diversity, is better for markers with more than two alleles, because it avoids impacts of within-population diversity when estimating genetic differentiation among populations (Jost 2008).

Landscape genetics

Landscape genetics was first defined as an "amalgamation of molecular population genetics and landscape ecology" (Manel et al. 2003). This field combines advanced molecular tools with landscape ecology and spatial statistics to investigate and quantify the effects of landscape variables (e.g. barriers, composition, configuration and habitat quality) on micro-evolutionary processes, i.e. gene flow and selection (Holderegger and Wagner 2008, Jaquiéry et al. 2011). It usually requires two key steps (Manel et al. 2003): 1) population genetics to detect discontinuities between populations or individuals; and 2) spatial correlation to identify effects of landscape features. Results provide information for further investigation in other disciplines including ecology, evolution, conservation, wildlife management and epidemiology (Zannèse et al. 2006, Neel 2008, Root et al. 2009, Wang et al. 2009, Goldberg and Waits 2010).

The rapid development of molecular genetics and technologies in spatial analysis has led to a rapid increase in landscape genetic studies in recent years. Up to 2008, there were over 600 articles dealing with landscape genetics published in various journals (Storfer et al. 2010). These articles have addressed a wide variety of research questions on a large range of taxa including vertebrates, invertebrates, plants, bacteria, viruses, lichens and fungi (Storfer et al. 2010). Five major research categories were identified through these publications (Storfer et al. 2007): (1) quantifying the influence of

landscape variables and configuration on genetic variation; (2) identifying barriers to gene flow; (3) identifying source-sink dynamics and movement corridors; (4) understanding the spatial and temporal scales of an ecological process; and (5) testing species-specific ecological hypotheses.

Compared with traditional population genetics, landscape genetics explicitly tests the effects of landscape heterogeneity on genetic structuring within and among populations (Holderegger and Wagner 2008, Storfer et al. 2010). Existing population genetic models assume large population sizes with high degrees of spatial symmetry (Segelbacher et al. 2010). Simple isolation-by-distance (IBD) models including only geographical distances between occupied habitat fragments have been demonstrated to be inadequate for explaining variations in genetic distance (e.g. Spear et al. 2005, McRae 2006, Spear and Storfer 2008, Murphy et al. 2010). Movements of animals may not only be limited by the spatial separation of habitat patches, but also by other landscape features, for example, potential barriers (rivers, high mountains, anthropogenic constructions, etc.), habitat transformation (agriculture, timber forests, etc.), or climatic characteristics (temperature, precipitation, humidity, etc.). Thus, multivariate models that take into account arrays of landscape characteristics to analyse the cause of population genetic structuring are becoming increasingly important to population genetic studies (Holderegger and Wagner 2008, Balkenhol et al. 2009). There have been no studies conducted to examine the association between landscape features and population genetic structures of the Australian granivores up to date.

THESIS JUSTIFICATION

Northern Australia has undergone substantial landscape changes through grazing, altered fire regimes and land clearing for agricultural purposes. Such changes may have contributed to the decline of approximately one-third of granivorous bird species of the region (Garnett and Crowley 2002). Many species of granivorous birds in northern Australia are sedentary (Higgins et al. 2006). Although the relationship between mobility and genetic structure has not been quantified, the population structure and genetic diversity of these species is more likely to be affected by landscape changes than that of other migratory species. This is because species that are highly dispersive are more likely to find and exploit new patches of resources when local environments become less favourable (Thomas 2000, Bergerot et al. 2012).

This research investigates the genetic diversity and structure of one granivorous bird species, the endangered Black-throated Finch, in response to landscape structure over multiple scales in northern Queensland, Australia. Applying molecular tools, my project is the first study to examine the population genetic structuring of a granivorous bird in relation to landscape features in Australia. It has improved the knowledge of the endangered Black-throated Finch in several critical areas, including: population genetic diversity, phylogenetic relationships between subspecies and conservation values of captive populations. Such genetic information is important to the understanding of the ecological impacts of landscape changes and to to the identification of genetically distinct populations relevant to conservation. It has also provided a means to recognise suitable habitats for the maintenance and persistence of the species.

RESEARCH AIMS

My thesis uses population and landscape genetics as well as phylogenetic approaches to answer a series of questions at local to regional scales, and at population, subspecies and species levels. The overall aims of my project are to:

1. Develop species-specific molecular tools to study the population genetics of the Black-throated Finch.
2. Examine the genetic structure and diversity of the Black-throated Finch at several locations across its range in northern Queensland, Australia.
3. Examine the relationship between current landscape features and the genetic structure of the Black-throated Finch at a fine spatial scale (in proximity of a man-made dam in northeastern Queensland).
4. Examine and compare genetic structure and diversity of wild and captive populations of the Black-throated Finch to determine the conservation values of the captive birds.

THESIS OUTLINE

In Chapter II, I describe field sampling protocols and laboratory procedures that were used to generate genetic data for the analysis in subsequent chapters. The number of samples and type of molecular markers used in each analysis are also explained in this chapter.

In Chapter III, I illustrate the development of novel microsatellite markers used for the Black-throated Finch using next-generation (454 shotgun) sequencing technology. Cross-species amplification was also performed on two related and co-occurring

species, the Double-barred Finch (*Taeniopygia bichenovii*) and the Chestnut-breasted Mannikin (*Lonchura castaneothorax*) to determine the value of these markers in other closely related species.

In Chapter IV, I evaluate the genetic diversity and structure of four populations of the Black-throated Finch. These four populations represent the majority of the birds known to be surviving across its range. Specifically, I estimated the richness and heterozogosity of both mitochondrial and microsatellite markers; examined the level of inbreeding; and evaluated the possibility of genetic bottleneck within each population. Bayesian clustering analysis was used to determine broad scale structuring among populations of the Black-throated Finch.

In Chapter V, I use a landscape genetic approach to examine the fine scale genetic structuring within the Townsville population of the Black-throated Finch in relation to various landscape variables. Environmental niche modelling was used to generate a landscape resistance matrix, which was then used to correlate with a genetic distance matrix.

In Chapter VI, I evaluate the conservation value of captive populations of the Black-throated Finch. Specifically, I sampled birds from various aviculturists and compared them with wild populations with respect to genetic diversity, population structuring, individual inbreeding, relatedness and presence of genetic bottleneck. I also performed individual assignments to determine possible origins of the captive birds with unknown ancestry information.

In Chapter VII, I synthesise overall findings from each chapter and highlight the key results that are important to the conservation of the Black-throated Finch. I also discuss implications for future studies and limitations of this research. Finally, I make detailed

recommendations for the conservation management of the Black-throated Finch based on the genetic knowledge developed in this research.

CHAPTER II

Field and laboratory procedures

(Photo credit: L. Stanley Tang)

INTRODUCTION

I collected samples for the analysis in each chapter in the same manner and followed the same laboratory procedures to extract DNA, to run polymerase chain reactions (PCRs) and to genotype all individuals. In this chapter, I detail the common procedures I have used both in the field and in the laboratory.

FIELD SAMPLING PROCEDURE

Sampling sites

On the basis of recent sightings records, I selected sites across the current range where the Black-throated Finch (*Poephila cincta*) had been seen regularly since 2009. The southern form (*P. c. cincta*) was sampled in the Townsville region (19.4333°S, 146.7667°E) in northern Queensland as well as in the Desert Uplands (22.1167°S, 146.4500°E) in central Queensland. The northern form (*P. c. atropygialis*) was sampled at Mareeba (16.9167°S, 145.3500°E) in far north Queensland. Samples for Lakefield National Park (14.8184°S, 144.2113°E) on Cape York Peninsula were obtained from a previous study (Maute 2011). I have also obtained additional samples of the same study by K. Maute from the Townsville region. All samples from the wild were collected between 2009 and 2013 (Figure II-1 and Table II-1).

I sourced samples of captive birds from aviculturists who keep records of each individual bird they possess through the Queensland Finch Society, the Finch Society of Australia and the Hunter Valley Finch Club in the states of Queensland and New South Wales in Australia (Appendix F). Each finch owner collected blood samples of their own birds following the same FTA© card protocol as mentioned below.

In Chapter III, only a subsample of the Townsville population was used for microsatellite marker development. In Chapter IV, all wild samples were used to examine the range-wide genetic diversity and structure. In Chapter V, the fine-scale landscape genetic analysis used only individuals sampled in the Townsville region and in Chapter VI, all wild and captive birds were used for the comparative analysis to infer conservation values of the *ex situ* populations (numbers of individuals used for each analysis are specified in relevant chapters).

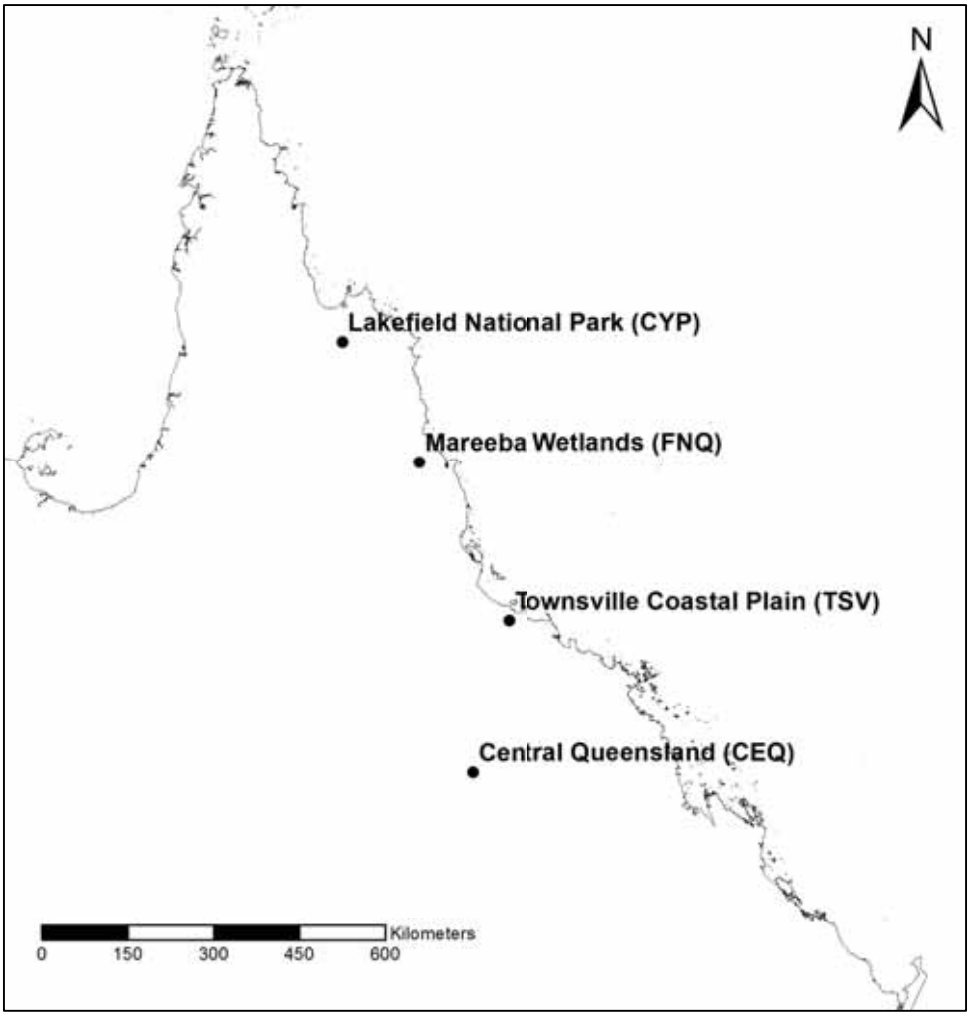

Figure II-1 Sampling locations of the wild Black-throated Finch (*Poephila cincta*) in Queensland, Australia.

Table II-1 Sampling sizes of the Black-throated Finch (*Poephila cincta*) from wild and captive populations in Australia

Region (wild)/Origin (captive)	Population code	Source	Sample size
Townsville Coastal Plain, North Queensland	TSV	Wild	86 + 48*
Desert Uplands, Central Queensland	CEQ	Wild	44
Mareeba Wetlands, Far North Queensland	FNQ	Wild	16
Lakefield National Park, Cape York Peninsula	CYP	Wild	48*
Subtotal			242
Armidale, North New South Wales	NSW	Captive	2
Murgon, Southeast Queensland	SEQ	Captive	22
Rockhampton, Central Queensland	ROC	Captive	18
Ayr, North Queensland	AYR	Captive	6
Unknown origin	UNK	Captive	49
Subtotal			97
TOTAL			339

* Samples collected by Kim Maute in 2009, 48 from TSV and 48 from CYP

Sample collection

The scarcity and patchy distribution of the Black-throated Finch makes it difficult to sample outside of a few known areas. However, the finch requires water daily, so restricted water availability in the dry season provides the best opportunity for capture. Typically, at each site I located possible watering points, e.g. dams, waterholes, creeks or farm troughs. Once birds were observed at these watering points, I set up mist-nets (mesh size 16 × 16mm, length 12m, 5 shelves; ECOTONE, Poland) nearby for two consecutive days. Two to ten nets were used depending on the size and shape of the watering points. All nets were generally opened half an hour before sunrise and closed before noon, and were checked every 15 – 20 minutes. Each bird trapped in mist-nets was immediately removed by licenced personnel and carefully placed in a breathable cloth bag. It was important that each bag only contained one individual to minimise the

risk of disease transmission. I then put a metal band on the right tarsus of each bird for future identification. These bands have unique codes and were supplied by the Australian Bird and Bat Banding Scheme (ABBBS). All by-catch species were released after banding. Blood samples were collected from all trapped Black-throated Finches following a standard procedure (Appdendix A). For samples provided by K. Maute, red cells were separated from fresh blood and stored in 90% ethanol instead of absorbing whole blood with FTA© cards (Maute 2011).

LABORATORY PROCEDURE

DNA extraction

DNA was extracted from blood spots on FTA© cards and from blood cells using ISOLATE Genomic DNA and ISOLATE II Blood DNA kits (Bioline, Australia), respectively. I modified standard protocols by increasing incubation times recommended by the manufacturer to yield large amounts (typically between 5-10μg) of purified DNA (Appendix B and C). DNA quality and quantity were assessed by electrophoresis (3V/min) on 1% agarose-TBE gels stained with GelGreen™ (Biotium, Australia), followed by quantification using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Australia). I then diluted purified DNA extractions with nuclease-free water to a standard concentration of approximately 5ng/μL for downstream analysis.

Microsatellite genotyping

All individuals were genotyped at 18 microsatellite loci developed specifically for the Black-throated Finch (Tang et al. 2014) (also see Chapter III). I performed three

multiplex PCRs using Type-it Microsatellite PCR kit (Qiagen, Australia) following Tang et al. (2014) (see Chapter III), with the cycling conditions: initial 5 minute denaturation at 95°C, 30 cycles of 95°C for 30 seconds; 60°C for 90 seconds and 72°C for 30 seconds, with a final 30 minute extension at 60°C.

I purified PCR products using MicroCLEAN DNA Clean-up reagent (Microzone, UK), following manufacturer's instructions (Appendix D). DNA fragments were then separated on an Applied Biosystems 3730xl Analyser with a LIZ600 internal size standard at Georgia Genomic Facility (Athens, USA) and scored based on fragment sizes using GENEMARKER version 2.2.0 (SoftGenetics, USA).

Mitochondrial DNA sequencing

In Chapter IV, in order to evaluate the effects of female-biased dispersal on the genetic structure, I amplified a 396 base-pair segment of the mitochondrial DNA control region in 5 individuals from each sampling region to represent the broad geographic area. The exception to this was for the Townsville population where I selected 10 individuals to account for multiple sampling locations in the area. I used previously published primers paCRL1 (5' -TGT-AGG-ATA-GCC-AAT-GTC-ATA-CG) and paCRH1 (5' –CGC-CTG-AAG-CCA-ATA-ACC-TA) (Rollins et al. 2012). These primers were designed for the Long-tailed Finch (*Poephila acuticauda*) from the consensus sequence between the Zebra Finch and the Black-throated Finch (Rollins et al. 2012). Each PCR reaction (20μL) for the mtDNA sequence contained approximately 10ng of genomic DNA, 0.2μM of each primer (forward and reverse) and 1× Type-it Multiplex PCR Master Mix (Qiagen, Australia). All samples were amplified in a C1000 Thermal Cycler (Bio-rad, Australia) under the following PCR conditions: initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds; annealing at 53°C for 90

seconds; extension at 72°C for 30 seconds, with a cycle of final extension at 60°C for 30 minutes. I checked the quality and quantity of PCR products with electrophoresis (1.3V/min) on 1.5% agarose-TBE gels stained with GelGreen™. Purification of the PCR products and the Big Dye Terminator (BDT) sequencing reactions were completed at the Australian Genomic Research Facility (AGRF), Brisbane, Australia. Products of the BDT reactions were ethanol precipitated and run on an Applied Biosystems 3730xl capillary sequencer at AGRF. All samples were sequenced in both the forward and reverse directions to ensure accuracy of nucleotide identification.

I further screened all sequences in 4PEAKS version 1.7.2 (Nucleobytes Inc., The Netherlands) for any misidentified base pairs in the sequences. Accepted sequences were then aligned in MEGA version 6.06 (Tamura et al. 2013) and trimmed to a uniform length of 302 base pairs. This sequence matched with the previously identified control region sequence of other close-related species: *P. acuticauda* (99% match, GenBank #JQ255398) and *Erythrura gouldiae* (87% match, GenBank #EF094896) using the Basic Local Alignment Search Tool (BLAST – http://www.ncbi.nlm.nih.gov/blast/). All 25 sequences were then submitted to GenBank with accession numbers from KY610021 to KY610045.

CHAPTER III

Development, characterisation and cross-species amplification of 18 novel microsatellite markers for the endangered Black-throated Finch (*Poephila cincta*) in Australia[1]

(Photo credit: L. Stanley Tang)

[1] This chapter is published as:

Tang, L. S., C. Smith-Keune, M. G. Gardner, and B. D. Hardesty. 2014. Development, characterisation and cross-species amplification of 16 novel microsatellite markers for the endangered Black-throated Finch (*Poephila cincta*) in Australia. Conservation Genetics Resources **6**:143-146.

ABSTRACT

The Black-throated Finch (Southern) (*Poephila cincta cincta*) is threatened by the substantial landscape changes in northern Australia. I developed 18 polymorphic microsatellite markers using 454-shotgun whole-genome sequencing technology. I identified an average of 4.7 alleles per locus based on 63 wild caught individuals from Townsville, Queensland. Thirteen and 9 markers were also successfully cross-amplified in two confamilial species, the Double-barred Finch (*Taeniopygia bichenovii*) and the Chestnut-breasted Mannikin (*Lonchura castaneothorax*) with 11 and 5 were polymorphic, respectively. These markers will help understand the population genetic structure of the endangered Black-throated Finch and determine genetic consequences of landscape changes for the species.

INTRODUCTION

Approximately one-third of granivorous birds in northern Australia are in decline due to the substantial landscape changes in the region (Franklin et al. 2005). The Black-throated Finch (*Poephila cincta*) is one such declining species. In Australia, it is listed as "Endangered" under the *Environmental Protection and Biodiversity Conservation Act 1999* (Commonwealth) nationally; as "Presumed Extinct" under the *Threatened Species Conservation Act 1995* in the state of New South Wales; and as "Vulnerable" under the *Nature Conservation Act 1992* in the state of Queensland.

There is little information on the population genetic structure and connectivity for most of granivorous birds in Australia. Although landscape changes reduce the suitability of the habitats of many granivorous birds, the lack of genetic information makes it difficult to accurately examine the potential evolutionary impacts of human related habitat changes on this fast declining group of birds. Limited marker are available for a few species of granivores, e.g. the Zebra Finch (*Taeniopygia guttata*) (Forstmeier et al. 2007) and the Long-tailed Finch (*Poephila acuticauda*), (Rollins et al. 2012). High-resolution markers are still lacking for most of the species.

Microsatellite DNA or simple sequence repeats are DNA sequences that contain repeated motifs of one to six bases. They are found in all prokaryotic and eukaryotic genomes with high levels of length polymorphism. Microsatellite markers have been widely used in many fields including gene mapping, forensic studies, population genetics and conservation biology (Zane et al. 2002). The development of these markers traditionally involves isolation from partial genomic sequences of the target species and screening several thousands of clones through hybridisation with repeat-containing probes (Rassmann et al. 1991). Such traditional isolation protocols are laborious, costly

and inefficient, due to the fact that they need to be isolated *de novo* for most species. Consequently, the strategy of designing "universal primers" is more problematic limiting their potential (Zane et al. 2002). For example, in birds, the success rate of cross-species amplification and polymorphism detection is only around 50% in species that diverged between 10 and 20 million years ago (Primmer et al. 1996).

New technologies that utilise low-coverage whole-genome sequencing have emerged as fast and cost-effective methods of isolating large numbers of markers suitable for population and evolutionary genetic studies. One of these methods in particular, the next generation sequencing, is in the process of taking over from the traditional protocols as the preferred means for microsatellite developments, as it is particularly beneficial for conservation biologists working with non-model taxa (Abdelkrim et al. 2009, Castoe et al. 2010). This method has several advantages over traditional microsatellite development. First, the chance of missing out possible repeat motifs can be greatly reduced, because traditional methods require only a few commonly identified repeat motifs and they can vary widely amongst taxa (Dieringer and Schlotterer 2003). Second, the confounding effects (over representation of fragments containing other repetitive elements) of the recovered loci can be avoided because next generation sequencing does not require the use of restriction enzymes to cut the DNA into fragments, hence eliminating the chance of such enzymes cutting within transposable elements of the DNA (Meglecz et al. 2007). Last, there is no need to edit sequence chromatograms and it is quicker, cheaper and recovers more loci (Gardner et al. 2011).

In this chapter, I describe the development of 16 polymorphic nuclear microsatellite markers for *P. c. cincta* using next generation sequencing technology. These markers are useful to quantify population connectivity of *P. cincta* and to investigate the genetic

consequences of landscape changes. I also described preliminary tests of cross-species amplification of these markers on two confamilial species, the Double-barred Finch (*Taeniopygia bichenovii*) and the chestnut-breasted Mannikin (*Lonchura castaneothorax*).

METHODS

The Black-throated Finch was sampled for next generation sequencing from the Townsville region in North Queensland, Australia. I extracted and purified genomic DNA (5µg) from one blood sample of *P. c. cincta* stored on Classic FTA® cards (Whatman, USA) using ISOLATE Genomic DNA kits (Bioline, Australia) as per manufacturer's instructions. The DNA was shotgun sequenced on Roche 454 GS-FLX instrumentation at Australian Genomic Research Facility, Brisbane, following Gardner et al. (2011).

I screened the resulting sequences for perfect and compound microsatellite loci (di- to hex-nucleotide motifs) with six or more repeats using the default settings of QDD version 2.0 (Meglecz et al. 2010). Primers were designed in QDD version 2.0, using a published PERL script that incorporates PRIMER3 software according to the following parameter restrictions: pure repeats, primer size between 18 and 22, GC% between 40 and 60, PCR product size between 100 and 500bp, high 3' stability and similar melting temperature. I then synthesised the primers for the 40 highest quality loci through Life Technologies Australia, with 5' tailing (5'-GGTGGCGACTCCTGGAG-3') to enable indirect fluorescent labelling and to minimise costs.

Initially, I tested all 40 loci for amplification success and specificity in eight individuals of *P. c. cincta* from geographically distinct locations, using the Type-it Microsatellite

PCR kit (Qiagen, Australia). I performed individual amplifications in 10μL reaction containing 4-6ng DNA template, 1×Type-it Multiplex PCR Master Mix (Qiagen, Australia), 0.2μM each primer (forward and reverse). Indirectly labelled reactions contained the tailed forward primer and a reporter primer (5' labelled with fluorescent dye modification 6-FAM, VIC, NED and PET) at a 1:4 ratio (total = 0.2μM). All loci were amplified in a C1000 Thermal Cycler (Bio-Rad, Australia) under the following PCR conditions: initial 5 minutes of denaturation at 95°C, 30 cycles of 95°C for 30 seconds; 60°C for 90 seconds; 72°C for 30 seconds, with a final 30 minutes of extension at 60°C. PCR products were visually checked for amplification success and whether products were of expected sizes by electrophoresis through 2% agarose gel. I then purified PCR products using MicroCLEAN DNA Clean-up Reagent (Microzone, UK), following manufacturer's instructions. DNA fragments were separated on an Applied Biosystems 3730xl Analyser with a LIZ600 internal size standard at Georgia Genomic Facility (Athens, USA) and scored using GeneMarker version 2.2.0 (SoftGenetics, USA).

I synthesised directly fluorescent-labelled forward primers (6-FAM, VIC, NED and PET) through Life Technologies Australia, for the selected loci to allow PCR multiplexing of up to six loci (Table III-1). I genotyped these markers in multiplex reactions for 63 *P. c. cincta* individuals caught near Townsville, Queensland. Multiplex combinations were designed using Multiplex Manager 1.2 (Holleley and Geerts 2009) and tested using PCR conditions described above with varied primer concentrations (Table III-1).

I calculated number of alleles (N), observed (H_O) and expected heterozygosities (H_E), and polymorphic information content (PIC) for each locus in CERVUS version 3.0

(Kalinowski et al. 2007). I tested for deviations from Hardy-Weinberg Equilibrium (HWE) in GenAlEx version 6.5 (Peakall and Smouse 2012). Sequential Bonferroni corrections were used to correct for multiple comparisons (Benjamini and Hochberg 1995). I also investigated potential linkage disequilibrium between pairs of loci using GENEPOP version 4.2 (Rousset 2008) and screened for potential null alleles in MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004).

I also tested all 18 loci in eight individuals of *T. bichenovii* and five individuals of *L. castaneothorax* from the Townsville region. A positive control containing DNA from the original species was used in each experiment. I extracted DNA from blood samples of both species using the same protocol as described above. All PCR and genotyping protocols of cross-species testing were the same as above.

RESULTS AND DISCUSSION

The initial shotgun sequencing produced 128,854 reads with an average length of 285bp and total GC content of 42.7%. There were 1,177 microsatellite loci identified after the initial screening in QDD version 2.0 and PCR primers were successfully designed for all.

Genotyping of the 40 best microsatellite loci resulted in 18 loci that had interpret-2 peak profiles. I found all these 18 loci were in HWE, except for btfi27, and one pair of loci (btfi38 and btfi40) exhibited significant linkage disequilibrium after Bonferroni corrections. Null alleles may be present at loci btfi04 and btfi27 as indicated by homozygote excess. Marker btfi23 contained one base pair alleles possibly due to indel mutations in the flanking region. Loci 31 and 31-2 were co-amplified and possibly contain over lapping alleles. *P. c. cincta* polymorphic markers exhibited 2 to 11 alleles

per locus with an average of 4.7 (± 0.72 standard error). All markers displayed an average expected heterozygosity of 0.47 (± 0.075 standard error).

Lengths of loci in cross-species amplifications were typically very similar across the species tested. Amplification was high for all multiplex suites, although levels of polymorphism varied. Thirteen loci amplified successfully for *T. bichenovii*, and 11 were polymorphic with 2 to 9 alleles per locus (Table III-2). Nine loci amplified successfully for *L. castaneothorax* and 5 were polymorphic with 2 to 5 alleles per locus (Table III-2). Transferability of *P. cincta* markers was moderately high (11 out of 18) in *T. bichonovii*, indicating these markers are likely to be informative for population studies within the genus *Taeniopygia*. On the contrary, the low level of transferability (9 out of 18) of these markers in *L. castaneothorax* suggests their limited suitability for population studies in the genus *Lonchura*. This highlights the potential utility of these markers across other related grassfinch species (Estrildidae), although further optimisation may be required.

Tropical and subtropical grassy woodlands are under increasing threat from habitat loss and fragmentation as a result of agricultural, industrial and urban development in northern Australia. The Black-throated Finch that utilises such habitats faces continuing population decline. The newly developed microsatellite markers herein will provide a useful tool to examine the genetic structure and connectivity, to monitor the genetic diversity, and to detect inbreeding depression in the species so that appropriate conservation management strategies can be implemented.

Table III-1 Details for 18 Black-throated Finch (*Poephila cincta cincta*) microsatellite loci developed from 454 shotgun sequences

Locus	Repeat motif	Primer sequence (5' – 3')	Primer conc. (µM)	Size range (bp)	*N*	N_A	H_O	H_E	P_{HWE}	*PIC*	GenBank accession no.
btfi01[B]	$(AT)_7$	[VIC]CAAGCAGTCACAGAACAACCA GACATATCCAAGGAAGCCAAA	3	108-116	63	2	0.032	0.031	0.898	0.031	KF366586
btfi02[C]	$(ACG)_9$	[PET]GTTGTTGGTCTGCTGGGACT CCTCTGACAGCAGGACAAGG	4	105-120	63	6	0.667	0.736	0.082	0.698	KF366587
btfi04[B]	$(AG)_7$	[NED]TGTCTGCAGCACTTCCAAAG AGACCATCTGGAGCTTGTGC	3	201-207	62	4	0.387	0.534	0.007	0.477	KF366588
btfi05[A]	$(AGT)_{10}$	[PET]CCAAATGGGACAGAGTGGAT CTCAGCCTCGCTTTGCTTC	5	169-217	54	10	0.685	0.734	0.999	0.707	KF366590
btfi06[C]	$(AG)_7$	[6-FAM]CTCAGCCACTCTGGCAAACT TTGGCCAGAGAATAAGCTGC	2	163-165	63	2	0.032	0.031	0.898	0.031	KF366591
btfi09[A]	$(AGC)_7$	[VIC]TTTGTGCCAGAAAGCTGGAT ACTCCTGAGCAGTGGTCCTG	3	123-129	54	3	0.648	0.593	0.608	0.512	KF366592
btfi22[A]	$(AAAT)_{10}$	[NED]TTTGAGAATGGCCTGGTTAGA CCTGCTTCCAGTTACCATGC	3	140-160	54	6	0.63	0.667	0.864	0.603	KF366594
btfi23[B§]	$(AC)_9$	[6-FAM]GAATGCTCCCACATCTCCTG CTCCTGTTTGCTGAGGGAAA	4	410-418	63	5	0.746	0.620	0.369	0.566	KF366600
btfi27[B]	$(AGAGGG)_7$	[PET]ACCCTGAGGAGTGGAAGGAG CCAAGCCTTGCCATCTATTG	3	146-200	60	6	0.350	0.659	0.000	0.6	KF366589
btfi31[C*]	$(AG)_{10}$	[PET]CCTCCACAAATGTCTAGGGAA ATCCCAGCATACTCCCGAA	4	214-226	60	2	0.067	0.065	0.789	0.062	KF366593
btfi31-2[C*]	$(AG)_{10}$	[PET]CCTCCACAAATGTCTAGGGAA ATCCCAGCATACTCCCGAA	4	238-246	63	2	0.063	0.062	0.795	0.06	KF366593
btfi34[A]	$(AAT)_6$	[6-FAM]TGGGTTATACAGCCCAGAGG CGTTGTCTGTCTTCAGTCCCA	1.5	168-171	54	2	0.222	0.199	0.358	0.178	KF366595
btfi36[A]	$(AAT)_6$	[6-FAM]GGCATTCCTGTCTTAGGAATCA GACACCGTCCTCCTGTAACG	3	337	54	1	-	-	-	-	KF366596
btfi38[B]	$(AAAAC)_6$	[6-FAM]GGCTGAGCTCTCACCAACAG CTTCAGGAGCACATTGAGCA	1.5	205-245	63	7	0.841	0.756	0.076	0.712	KF366597
btfi39[C]	$(ACAG)_6$	[6-FAM]GGAGCAGATGGAGAGCTGAG GGTGTCCTTCACTGTGTCCC	2	244-260	63	5	0.73	0.671	0.691	0.603	KF366598
btfi40[C]	$(ACCT)_7$	[VIC]TTGCATACATCAATGTCAGGTG TGCTTATCACCAAAGCAGCA	2	130-170	63	11	0.905	0.866	0.100	0.844	KF366599
btfi41[A]	$(AGG)_6$	[6-FAM]TCCTAGTTCTGGCAGACCCTT ATCTCTGTCATGTCGGTCCC	2	224-230	54	2	0.204	0.316	0.010	0.264	KF366601
btfi43[B]	$(AGT)_8$	[VIC]TCATCCACCTGTCACACCAG AGCAGCTCCAGTTGCCTGTA	2	323	63	1	-	-	-	-	KF366602

N – number of individuals amplified; N_A – number of alleles; H_O – observed heterozygosity; H_E – expected heterozygosity; P_{HWE} – significance value of Hardy-Weinberg Equilibrium at $P < 0.0033$ after FDR correction; *PIC* – polymorphic information content

[A, B, C] Multiplex systems

[*] Loci btfi31 and btfi31-2 were co-amplified with the same set of primers and may contain over lapping alleles

[§] Marker btfi23 contains out-of-phase (1 bp) alleles

Table III-2 Cross-species amplification success (number of scorable individuals out of total individuals amplified), allele size range in base pairs (bp) and number of alleles (N_A) for the novel Black-throated Finch (*Poephila cincta cincta*) microsatellite loci in the Double-barred Finch (*Taeniopygia bichenovii*) and Chestnut-breasted Mannikin (*Lonchura castaneothorax*)

Locus	Success	Size Range (bp)	N_A	Locus	Success	Size Range (bp)	N_A
	Taeniopygia bichenovii				*Lonchura castaneothorax*		
btfi01	0/8	-	-	**btfi01**	0/5	-	-
btfi02	8/8	105-114	4	**btfi02**	5/5	102-114	5
btfi04	8/8	199-201	2	**btfi04**	5/5	199-201	2
btfi05	7/8	181-235	9	**btfi05**	0/5	-	-
btfi06	8/8	163	1	**btfi06**	5/5	163	1
btfi09	8/8	120-123	2	**btfi09**	5/5	123	1
btfi22	0/8	-	-	**btfi22**	0/5	-	-
btfi23	7/8	406-416	4	**btfi23**	0/5	-	-
btfi27	6/8	134-170	5	**btfi27**	5/5	164-170	3
btfi31	7/8	212-218	3	**btfi31**	4/5	212-218	2
btfi31-2	0/8	-	-	**btfi31-2**	0/5	-	-
btfi34	8/8	168-180	4	**btfi34**	5/5	168	1
btfi36	5/8	337	1	**btfi36**	0/5	-	-
btfi38	0/8	-	-	**btfi38**	0/5	-	-
btfi39	0/8	-	-	**btfi39**	0/5	-	-
btfi40	6/8	158-190	6	**btfi40**	0/5	-	-
btfi41	8/8	227-239	5	**btfi41**	5/5	227	1
btfi43	8/8	311-326	4	**btfi43**	5/5	311-329	4

CHAPTER IV

Genetic structure and diversity of the Black-throated Finch (*Poephila cincta*) across its current distribution[2]

(Photo credit: L. Stanley Tang)

[2] This chapter is published as:

Tang, L. S., C. Smith-Keune, A. C. Grice, J. M. Moloney and B. D. Hardesty. In Press. Genetic structure and diversity of the black-throated finch (*Poephila cincta*) across its current range. Australian Journal of Zoology.

ABSTRACT

Understanding the patterns of population connectivity and level of genetic diversity can facilitate the identification of both ecologically relevant populations and the spatial scales at which conservation management may need to focus. I quantified genetic variation within and among populations of Black-throated Finches across their current distribution. To quantify genetic structure and diversity, I genotyped 242 individuals from four populations using 15 polymorphic microsatellite markers and sequenced 25 individuals based on a 302 base-pair segment of mitochondrial control region. I found modest levels of genetic diversity (average allelic richness $r = 4.37 \pm 0.41$ standard error and average heterozygosity $H_O = 0.42 \pm 0.040$ standard error) with no bottleneck signature among sampled populations. I identified two genetic groups that represent populations of two subspecies based on Bayesian clustering analysis and low levels of genetic differentiation based on pairwise genetic differentiation statistics (all F_{ST}, R_{ST} and Nei's unbiased D values < 0.1). Our data suggest that genetic exchange occurs among sampled populations despite recent population declines. Conservation efforts that focus on maintaining habitat connectivity and increasing habitat quality to ensure a high level of gene flow on a larger scale will improve the species' ability to persist in changing landscapes. I also suggest that conservation management should support continuous monitoring of the bird to identify any rapid population declines as land use intensification occurs throughout the species' range.

INTRODUCTION

Quantifying genetic diversity and connectivity in declining populations

Genetics contributes to conservation of biodiversity at both species and ecosystem levels (McNeely et al. 1990, Frankham 2010a). At the species level, genetic factors affect the risk of population or species extinction through inbreeding depression, loss of genetic diversity and decreased evolutionary potential (Frankham 2005). Genetic variability also contributes to the survival, functioning and diversity of all ecosystems (Reusch et al. 2005, Crutsinger et al. 2006). Therefore, applying molecular genetics to acquire vital information, including effective population size, demographic history, population structure, gene flow and genetic diversity, for species conservation has become one of the primary goals of conservation genetics (Frankham 2010a).

Small, isolated populations may have an increased extinction risk due to environmental, demographic and genetic stochasticity (Lande 1988). Habitat loss and fragmentation may lead to further isolation of small populations in habitat remnants, decreasing the viability of the metapopulation (Hanski and Ovaskainen 2000). For example, a lack of habitat connectivity and suitable breeding habitat in human impacted landscapes has led to the decline of the endangered Black-capped Vireo (*Vireo atricapilla*) in the USA. As a result, lower genetic diversity and increased differentiation have been observed in current populations compared with historical samples, indicating the significant impact of habitat fragmentation (Athrey et al. 2012).

Additionally, population fitness is likely to be lost both through increased inbreeding depression at the individual level and through loss of genetic diversity at the population level (Frankham 2005). Studies of the Glanville Fritillary Butterfly metapopulation in Finland, for example, have demonstrated that inbreeding contributes to the extinction of

wild populations. Inbreeding reduced the larval survival of the butterflies by reducing the egg hatch rate and increasing the pupal period, increasing the chance that pupae would be parasitised by wasps. Inbreeding also shortened the lifespan of the female, leading to reduced egg production (Saccheri et al. 1998). Overall, the adaptability of small, isolated populations to environmental changes is reduced through decreased population viability and fitness, hence increasing the extinction risk.

For populations of conservation concern, it is important to understand the pattern and degree of genetic connectivity and diversity, so that appropriate conservation management strategies can be implemented. For example, British populations of the Black Grouse (*Tetrao tetrix*) have become fragmented and isolated into three genetically distinct populations with little migration between them. The genetic differentiation observed is likely due to genetic drift in small and recently fragmented sub-populations. Given that isolation has occurred recently, it is appropriate to manage these populations as a whole (Höglund et al. 2011). Accurate estimation of genetic diversity may also help avoid adverse effects such as underestimated extinction risk, inappropriate recovery strategies (including reintroduction) and mixing of different evolutionarily significant units with reduced fitness (Frankham 2005). For example, in the absence of genetic data, the reintroduced populations of the Koala (*Phascolarctos cinereus*) in south-eastern Australia were from a highly inbred island population. As a result, they have suffered low genetic diversity and fitness, including poor sperm quality and high frequency of testicular aplasia (Seymour et al. 2001).

Habitat change and the decline of granivorous birds in Australia

In Australia, habitat alteration and landscape changes have resulted in the decline of many faunal groups, including granivorous birds, and appropriate conservation

management strategies are urgently needed to ensure population and species persistence (Lindenmayer 2009). Granivorous birds represent more than a quarter of all threatened land bird taxa in Australia (Garnett and Crowley 2000b), and substantial landscape changes have resulted in the dramatic decline in these birds in recent decades. The tropical and subtropical savannahs of northern Australia, which support the largest granivorous bird assemblages, have experienced the greatest declines (State of the Environment Advisory Council 1996, Franklin et al. 2005).

The Black-throated Finch (*Poephila cincta*) is a granivorous bird endemic to the mixed woodlands habitat of Australia. Since 1998, the southern form has not been seen from central to north Queensland and extensive field surveys and sighting records suggest that the species may have declined by 50% in the past 20 years (Environment Australia 2000, Black-throated Finch Recovery Team 2007). The decline of the Black-throated Finch began early in the 1900s with the expansion of pastoralism into its habitat and is associated with substantial landscape changes in the region (Franklin et al. 2000).

The movement patterns of the bird are poorly known. The available evidence from surveys and sighting records suggest that the bird is sedentary with only limited movements in response to food availability or post-breeding dispersal (Baldwin 1976, Mitchell 1996, Natural Resource Assessment Environmental Consultants 2007, Forshaw et al. 2012). In addition, based on recorded sightings, the distribution of *P.c. cincta* now appears to be severely fragmented and populations continue to decline. It is therefore likely that the existing populations of the bird are at risk of losing genetic diversity with potentially negative implications for adaptation to future environmental changes.

In this study, I provide the first quantitative measure of the pattern and degree of population connectivity and genetic diversity of the Black-throated Finch across its current range. Specifically, I examined the genetic diversity of four finch populations from central Queensland, northern Queensland, far northern Queensland and Cape York Peninsula. I also used individual and population based approaches to delineate the genetic structure and to estimate the level of gene flow within and among these populations. I hypothesised that: (1) genetic diversity within populations is low; (2) recent population contraction/genetic bottleneck is likely to be present; (3) populations are likely to show distinct genetic structures with low levels of gene flow between them; and (4) genetic differentiation between two subspecies is likely to be significant.

METHODS

Field sampling and genetic procedures

Sample collection

On the basis of sightings records in the past five years, I sampled sites across the current range where the Black-throated Finch had been seen regularly (Chapter II). The southern form (*P. c. cincta*) was sampled in the Townsville region as well as in the Desert Uplands in central Queensland. The northern form (*P. c. atropygialis*) was sampled at Mareeba in far north Queensland. I collected small amount of blood (30-50μL) from each individual trapped using mist nets following strict protocols. Blood samples were applied to FTA© cards and dried for further analysis (Appendix A). Samples from Lakefield National Park on Cape York Peninsula were obtained from a previous study (Maute 2011) and were stored as blood cells in 70% ethanol.

DNA extraction and genotyping

DNA was extracted from blood spots on FTA© cards and from blood cells using ISOLATE Genomic DNA and ISOLATE II Blood DNA kits (Bioline, Australia), respectively. Protocols were modified by increasing incubation times as recommended by the manufacturer to yield large amounts (5-10μg) of purified DNA (Appendix B and C).

All individuals were genotyped at 18 microsatellite loci developed specifically for the Black-throated Finch (Tang et al. 2014). Microsatellite genotyping procedure is detailed in Chapter II.

I amplified a 396 base-pair segment of the mitochondrial DNA control region in 5 individuals from each region to represent the broad geographic area. The exception to this was for the Townsville population where I selected 10 individuals to account for the genetic differentiation within this region. Details of the mitochondrial DNA sequencing are in Chapter II.

Statistical Analysis

Marker characteristics

All microsatellite genotype data were checked for evidence of typographic and scoring errors (large allele dropout, stuttering and the presence of null alleles) for each population using MICROCHECKER version 2.2.3 (Van Oosterhout et al. 2004). I also performed neutrality tests in POPGENE version 1.31 (Yeh and Boyle 1997) for microsatellite markers and in DnaSP version 5.10.1 (Librado and Rozas 2009) for mitochondrial DNA to confirm that all selected markers evolved neutrally across populations.

I analysed all microsatellite loci to test for departures from Hardy-Weinberg equilibria (HWE) within each population by performing global χ^2 goodness-of-fit tests across population-specific F_{IS} (inbreeding coefficient) estimates, and linkage disequilibrium (LD) between all pairs of loci in GENEPOP version 4.3 (Rousset 2008). I used Markov Chain Monte Carlo (MCMC) algorithms to estimate the probabilities of all tests with 10,000 dememorisation steps, 1,000 batches and 5,000 iterations per batch (Guo and Thompson 1992, Slatkin and Excoffier 1996). A sequential Bonferroni correction was also applied to all probability values of multiple comparisons to reduce Type I statistical error (Zar 1999).

Genetic variability

For microsatellite markers, I calculated the observed heterozygosity (H_O), the expected heterozygosity (H_E), and the number of private alleles (N_{PA}) in GenAlEx version 6.5 (Peakall and Smouse 2006, 2012). N_{PA} is often used to estimate the amount of gene flow (N_m, the number of migrants) between local populations (Slatkin and Barton 1989). The average allelic richness (number of alleles, *r*), allele frequencies and average genetic diversity (GD) were calculated in FSTAT version 2.9.3.2 (Goudet 1995). The method used to calculate *r* incorporates a rarefaction method to compensate for unequal sample sizes (el Mousadik and Petit 1996), as is appropriate given our sampling design. Population-specific F_{IS} values were calculated and tested for statistical significance by randomising alleles within populations in FSTAT version 2.9.3.2 in order to identify deviations from HWE and potential substructuring within populations (Wahlund 1928).

I examined genetic diversity for the mitochondrial DNA sequence by calculating the number of haplotypes (N_H), haplotype diversity (h) and nucleotide diversity (π) in DnaSP version 5.10.1. The haplotype richness (H_P) was estimated in CONTRIB version

1.02 (Petit et al. 1998) with a rarefaction method to compensate for unequal sample sizes. I also manually counted the number of haplotypes that were found in only one locality (private haplotypes, N_{PH}) to estimate the amount of gene flow between local populations.

I used two traditional *ad hoc* methods to test for signatures of recent genetic bottlenecks within each population based on microsatellite data. First, heterozygosity excess was tested assuming a two-phase mutation (TPM) model with 95% stepwise mutations, 5% multiple-step mutations, and a variance among multiple steps of 12, in BOTTLENECK 1.2.02 (Piry et al. 1999). The TPM model is considered to be the most appropriate for microsatellite data (Piry et al. 1999). Significance of heterozygosity excess over all loci was determined with a one-tailed Wilcoxon sign rank test as implemented in the program. Second, I used the M-ratio method developed by Garza and Williamson (2001) to calculate the ratio (M) of the total number of alleles to the range in allele sizes, and its critical value M_C (5% of values fall below M_C as determined by simulations). M and M_C were estimated using M_P_VAL and CRITICAL_M, respectively (Garza and Williamson 2001). I assigned three required parameters that were required for both programmes with recommended values: (1) pre-bottleneck $\theta = 4N_e\mu = 10$ (where N_e = effective population size, μ = mutation rate); (2) the percentage of one step mutations, $\rho_s = 0.9$; and (3) the mean size of non one-step mutations, $\Delta_g = 3.5$. Each set of simulations consisted of 10,000 iterations.

Genetic structure

I analysed the genetic structure of all populations using four complementary approaches to increase the reliability of detecting genetic signals. First, I performed analyses of molecular variance (AMOVA) using allele frequencies to examine the genetic structure

of four populations when grouped into two subspecies (*P. c. cincta*: TSV and CEQ; *P. c. atropygialis*: FNQ and CYP) and without grouping. The significance of the analysis was tested running 10,000 permutations in ARLEQUIN version 3.5.1.3. (Excoffier and Lischer 2010).

Second, pairwise genetic differentiation among 4 *a priori* Black-throated Finch populations was estimated using statistics based on both an infinite allele model (IAM) (F_{ST}) and a stepwise mutation model (SMM) (R_{ST}). Both statistics were calculated in ARLEQUIN version 3.5.1.3. The significance of F_{ST} values was tested running 10,000 permutations and corrected with a sequential Bonferroni method for multiple tests. There is no direct probability test for R_{ST}. I used the estimated N_m as an indirect measure to quantify the significance of differentiation (Slatkin 1995). Specifically, if $N_m \leq 1$, I considered there was no gene flow between two populations, whereas $N_m \geq 3$ indicated a high level of gene flow (Wang et al. 2001). I also calculated the unbiased genetic distance *D* (Nei 1978) to estimate the genetic relationship among populations.

Third, I assessed the population genetic structure and individual admixture based on microsatellite variation using the Bayesian clustering approach implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). This programme uses an MCMC algorithm to infer distinct genetic clusters (*k*), estimate allele frequencies in each cluster and allocate population memberships of each individual. I chose an admixture model, assuming allele frequencies are correlated among populations. Trial runs without location as prior produced weak genetic differentiation with no distinct clusters. Therefore, I used an individual-based approach with sampling locations as prior. The simulations were run for 10 replicates at each *k* value ranging from 1 to 10. I chose the upper limit of *k* to be greater than the number of sampled populations to account for any

distinct genetic clusters that might be present within each population. Each replicate consisted of a burn-in period of 100,000 MCMC steps, followed by 2×10^5 iterations. Trial runs indicated that run durations were sufficient for likelihood values to stabilise. The best supported *k* values were determined by posterior probabilities (P(*D*)) and Δ*k* method (Evanno et al. 2005) in STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt 2012). The clusters output of the independent runs of the best supported *k* values were permuted and aligned using the "Full Search" algorithm to minimise the effects of "label switching" and "multimodality" in CLUMPP version 1.1.2 (Jakobsson and Rosenberg 2007). The output files were then visualised in DISTRUCT (Rosenberg 2004).

Last, I constructed a haplotype genealogy for the mitochondrial DNA control region in HAPLOVIEW (Barrett et al. 2005) using the topology of most parsimonious trees constructed in DNAPARS implemented in PHYLIP package version 3.695 (Felsenstein 2005). I used bootstrapping as the resampling method with 500 replicates to produce the consensus tree. The aligned sequence of the control region of the Gouldian Finch (GenBank #EF094896) was used to produce a rooted network.

RESULTS

Marker characteristics

There was no evidence for scoring errors associated with stuttering, large allele dropout or null alleles at any of the 18 microsatellite loci scored. Neutrality tests confirmed that all of the microsatellite and mitochondrial DNA markers were neutral. Markers btfi43

and btfi36 were monomorphic and marker btfi23 contained 1base pair of out-of-phase alleles. Because of this, they were not used in further analysis. After Bonferroni corrections (corrected $\alpha = 0.0031$), H_O deviations from the Hardy-Weinberg Equilibrium (HWE) were detected in at least one population at 5 loci (btfi04, btfi27, btfi31-2, btfi40 and btfi41). However, only locus btfi27 showed consistent deviation across all populations and this locus was therefore excluded from further analysis. Linkage disequilibria of each pair of the remaining 14 loci were not detected within or among populations after Bonferroni corrections (corrected $\alpha = 0.0005$).

Genetic variability

Microsatellite markers showed a modest level of polymorphism. The number of alleles ranged from 2 (btfi36) to 14 (btfi05), with an average of 4.367 (± 0.412 standard error) alleles per locus over all populations. The highest number of private alleles was detected in the TSV population ($N_{PA} = 6$); whereas the CEQ and CYP populations had only 3 private alleles each. No private alleles were detected in FNQ probably due to the small sample size of the population. The observed heterozygosity ranged from 0.373 to 0.444, with an average of 0.417 (± 0.040 standard error) across all populations. The expected heterozygosity exhibits a similar level of variation with an average of 0.429 (± 0.039 standard error) across all populations. Both the genetic diversity index (GD) and the allelic richness (r) showed few differences between populations. The inbreeding coefficient (F_{IS}) was also relatively low in all populations, indicating no significant heterozygosity deficiency (Table IV-1).

The control region of the mitochondrial DNA sequence data identified 5 polymorphic sites with two types of transitions: A-G (site 89, 135, 221 and 281) and T-C (site 241). These polymorphic sites defined 6 haplotypes, only one of which was common across

all populations (Hap_BA). However, the frequencies of Hap_BA were not evenly distributed within populations; it was highest in TSV with a frequency of 0.44 across all individuals sampled and lowest in CEQ with a frequency of 0.13 across all individuals sampled. Three haplotypes were found only in one population (Table IV-2). The overall haplotype diversity (*h*) was 0.587 (± 0.022 standard error) and the overall haplotype richness was 2.664. The nucleotide diversity was low (π = 0.003 ± 0.001 standard error). All identified haplotypes are stored in GENBANK at accessions KR676617 – KR676622.

Table IV-1 Measurements of genetic diversity for each of the Black-throated Finch (*Poephila cincta*) populations. N_{PA}: number of private alleles; H_O: observed heterozygosity; H_E: expected heterozygosity; *r*: average allelic richness; GD: genetic diversity index; F_{IS}: inbreeding coefficient

Region	N_{PA}	H_O (SE)	H_E (SE)	*r* (SE)	GD (SE)	F_{IS}
TSV	6	0.41±0.08	0.42±0.08	3.98±0.67	0.45±0.08	0.04
CEQ	3	0.37±0.08	0.40±0.08	3.84±0.66	0.43±0.08	0.08
FNQ	0	0.44±0.07	0.43±0.07	3.57±0.63	0.47±0.07	0.01
CYP	3	0.44±0.08	0.46±0.08	4.18±0.75	0.50±0.08	0.04

Table IV-2 Counts of six identified mitochondrial haplotype types in each population of the Black-throated Finch (*Poephila cincta*). Relative frequencies within each population shown in brackets

Region	Hap_BA	Hap_BB	Hap_BC	Hap_BD	HapBE	HapBF
TSV	7 (0.7)	1 (0.1)	2 (0.2)	-	-	-
CEQ	2 (0.4)	-	-	2 (0.4)	1 (0.2)	-
FNQ	4 (0.8)	-	-	-	-	1 (0.2)
CYP	3 (0.6)	-	-	-	1 (0.2)	1 (0.2)

There was no evidence for a significant genetic bottleneck detected in any populations using either the *M*-ratio or heterozygosity excess methods. Across all populations, the *M* ratios ranged between 0.903 and 0.964. These ratios were consistently higher than the M_C values. One-tailed Wilcoxon sign rank tests showed no significant heterozygosity

excess in any of the populations (all probability values were greater than 0.05) (Table IV-3).

Table IV-3 Result comparison of the two *ad-hoc* bottleneck analysis in each of the Black-throated Finch (*Poephila cincta*) population. *M*: ratio of total number of alleles to the range of allele sizes; M_C: critical value of *M*; P_W: probability value of one-tailed Wilcoxon sign rank test for heterozygosity excess. For M ratio method, if $M < M_C$; and for Heterozygosity excess method, if $P_W < 0.05$, a recent bottleneck is likely to be present

Region	M	M_C	Presence of bottleneck (M ratio)	P_W	Presence of bottleneck (Heterozygosity excess)
TSV	0.9034	0.7513	No	0.7492	No
CEQ	0.9644	0.7147	No	0.9119	No
FNQ	0.9111	0.6534	No	0.5270	No
CYP	0.9350	0.7200	No	0.9161	No

Genetic structure

AMOVA results based on grouping populations of the Black-throated Finch into two subspecies revealed significant differences between subspecies ($P = 0.013 < 0.05$), between populations within subspecies ($P < 0.0001$) and within populations ($P < 0.0001$). In all cases, most of the genetic variation (94.57%) was observed within populations (Table IV-4). The significant genetic differences between population pairs were further supported by F_{ST} estimates after Bonferroni corrections (corrected $\alpha = 0.0083$), except for FNQ and CYP. However, differences were generally weak with low F_{ST} values (mean $F_{ST} = 0.045$, ranging between 0.014 and 0.071) indicating low neutral genetic differentiation between all of the populations. This is reflected in low R_{ST} values (mean $R_{ST} = 0.048$, ranging between 0.014 and 0.077) with high levels of gene flow inferred (mean $N_m = 15.08$, Table IV-4). Nei's unbiased genetic distance (*D*) showed that populations of TSV and FNQ were the most different, followed by FNQ and CEQ.

The TSV and CEQ populations were the most similar, followed by FNQ and CYP (Table IV-4).

Table IV-4 Analysis of molecular variance (AMOVA) grouping populations of the Black-throated Finch according to subspecies (*Poephila cincta cincta* and *P. c. atropygialis*).

Source of variance	df	Sum of squares	Variance components	Percentage of variation
Between subspecies	1	32.988	0.115	3.36 (F_{CT} = 0.034, p < 0.001)
Between populations within subspecies	2	19.010	0.070	2.04 (F_{SC} = 0.021, p < 0.001)
Within populations	480	1544.610	3.226	94.57 (F_{ST} = 0.054, p = 0.013)
Total	483	1596.608	3.410	100

Furthermore, the individual based Bayesian clustering analysis in STRUCTURE identified two distinct genetic clusters (k = 2, Figure IV-1) with *a priori* population information. Roughly, FNQ and CYP belonged to one cluster, TSV and CEQ another, mirroring the differentiation between two subspecies of the Black-throated Finch. The maximum posterior probability ln P(D) also corresponded to k = 2 when population priors were inferred (Figure IV-2). This eliminated the possibility of a single genetic cluster of all Black-throated Finch populations.

The parsimony network of haplotypes identified only one major haplotype group connected with 5 small haplotype groups diverged by only one transition each (Figure IV-3). The overall small number of haplotypes identified among selected individuals indicated low levels of genetic (haplotype) diversity. The unclear haplotype structuring suggested a lack of historical genetic isolation between the four groups of the black-throated finch.

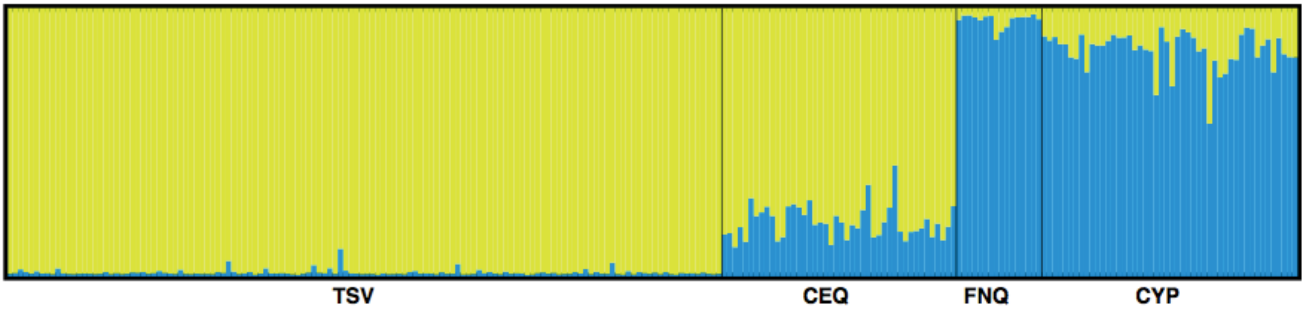

Figure IV-2 Individual membership coefficients derived from Bayesian inference of genetic structure across four Black-throated Finch populations using admixture model with (bottom, $k = 2$) *a priori* populations. A single vertical line represents an individual

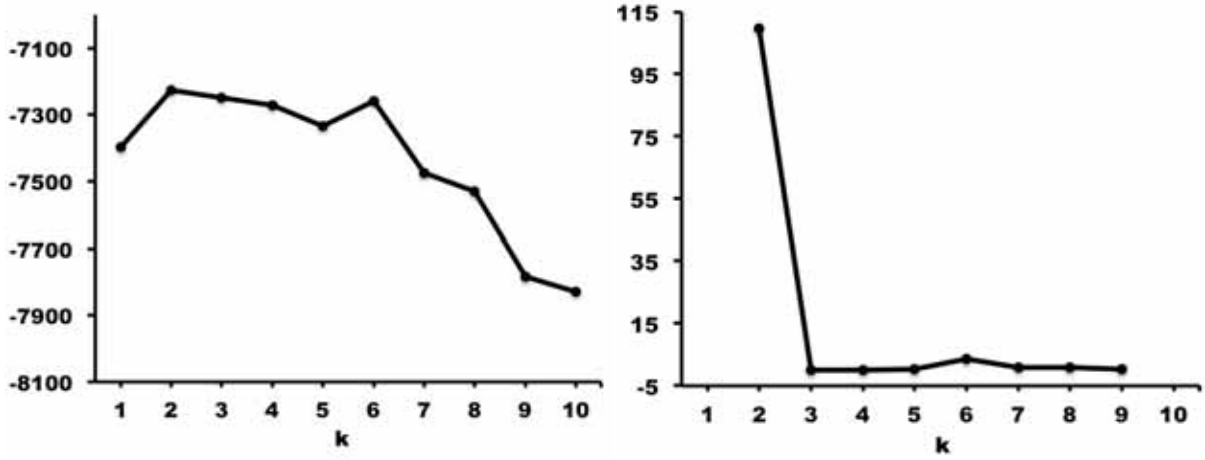

Figure IV-1 Log posterior probabilities (ln P(*D*), left) and Δ*k* (right) of the microsatellite data for each number of genetic clusters (*k*) tested

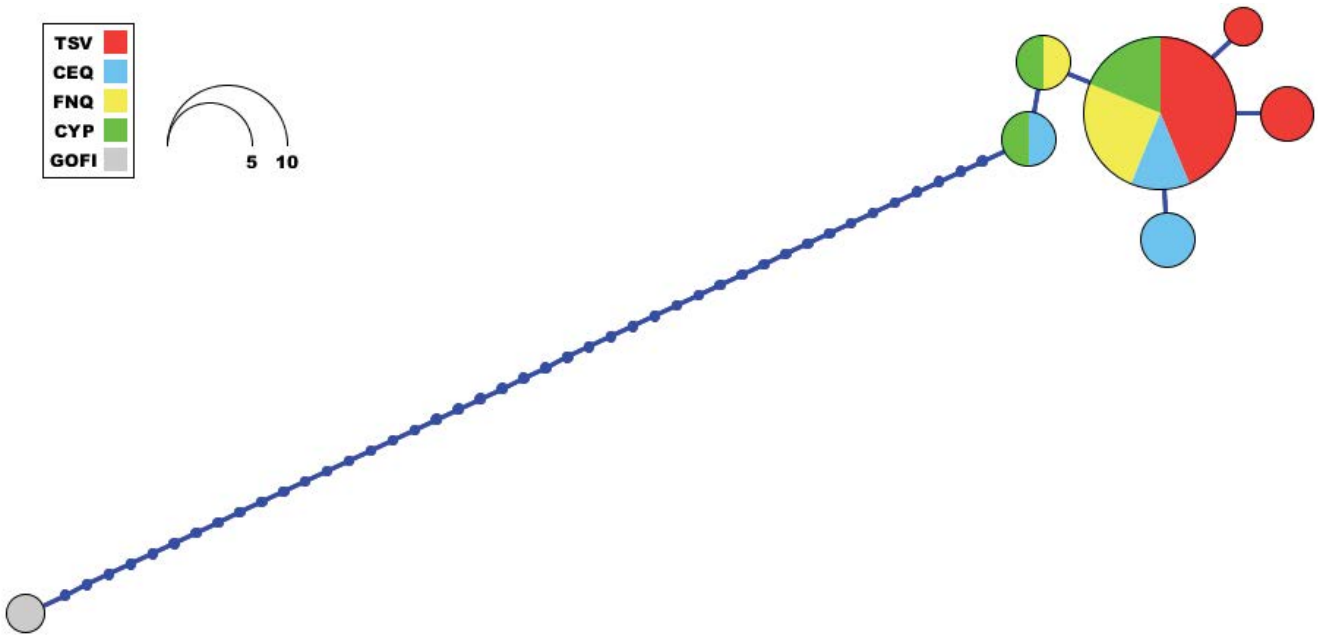

Figure IV-3 Mitochondrial DNA haplotype network of the selected Black-throated Finch samples from each population. Circle size indicates proportion of individuals assigned that haplotype. Solid lines connecting haplotypes are proportional to the number of mutations separating them. The network is rooted to the Gouldian Finch (GOFI)

DISCUSSION

To infer appropriate conservation management strategies, I examined the genetic diversity and structure of the endangered Black-throated Finch populations in Australia by using both microsatellite and mitochondrial DNA markers from more than 200 samples collected between 2009 and 2013. Genetic analyses provided evidence that the neutral genetic diversity within each population is modest. Genetic differentiation between two recognised subspecies was also observed, which has important management implications. This work provides information useful for the management and conservation of the endangered Black-throated Finch.

Genetic diversity

The levels of observed genetic diversity within the sampled populations of the Black-throated Finch are modest (*r* ranged from 3.57 to 4.18; H_O ranged from 0.37 to 0.44). This contrasts with the situation in the more common and widespread congener, the Long-tailed Finch (*Poephila acuticauda*), which on average has 16.4 alleles per locus and an average heterozygosity of 0.71 based on 12 microsatellite markers (Rollins et al. 2012). The use of both allelic richness and average heterozygosity identified that birds in TSV and CYP had a higher level of genetic diversity than those in CEQ and FNQ. Despite the small sampling size from CEQ, I have discovered several hundred Black-throated Finch in the region towards the end of the study. This population is probably larger than that from TSV. Therefore, there is no association between population sizes and the variation of genetic diversity.

The Black-throated Finch is considered sedentary and its current distribution is highly fragmented (Forshaw et al. 2012). Accordingly, neutral genetic diversity is expected to be lower than it would have been had the populations been mobile and in a continuous landscape, due to small or isolated populations resulting in stochastic loss of alleles and more rapid genetic drift. In birds, populations are generally considered to have low genetic diversity if the average number of microsatellite alleles is less than 3-4 and the average heterozygosity is below 0.5. For example, the endangered Black Robin (*Petroica traversi*) from New Zealand was reported to have one of the lowest heterozygosity values (0.2) among avian species, which confirmed the extreme low level of genetic diversity and history of severe population bottleneck of the species (Ardern and Lambert 1997). On the other hand, widely dispersed common species with high levels of genetic diversity, such as the House Sparrow (*Passer domesticus*) demonstrate allelic richness of 13.6 and average heterozygosity of 0.8 based on 8

microsatellite loci from multiple locations in North America, Europe and Africa (Schrey et al. 2011). Therefore, the levels of observed genetic diversity within sampled Black-throated Finch populations are modest compared with that of other species.

Genetic bottleneck

I hypothesised that genetic bottlenecks were likely to be present as a result of isolation and population declines. However, both heterozygosity excess and *M* ratio analyses identified no signature of bottleneck in the sampled Black-throated Finch populations. Many studies have suggested that endangered populations do not necessarily have to experience recent bottleneck events to show low levels of neutral genetic diversity. For example, the extremely low levels of genetic diversity in the Madagascar Fish Eagle (*Haliaeetus vocifer*) is due to historically small population sizes (over thousands of years) rather than recent population bottlenecks (Johnson et al. 2009). Similarly, other vertebrate species, including the Wandering Albatross (*Diomedea exulans*) (Milot et al. 2007), the Kangaroo Rat (*Dipodomys heermanni morroensis*) (Matocq and Villablanca 2001), and the Brown Bear (*Ursus arctos*) (Paetkau et al. 1998) also show low levels of genetic diversity without signatures of recent bottlenecks, in spite of experiencing recent habitat loss.

The continuance of low genetic diversity without signatures of recent bottlenecks is likely due to the accumulation of nucleotide substitutions by low annual fecundity of the species, or due to a reduced rate of mutation by increased body size and slower metabolic rates (Gillooly et al. 2005, Milot et al. 2007). However, the Black-throated Finch is a small bird that has a relatively fast metabolic rate with high annual fecundity. Its fast reproductive cycle also increases the rate of mutation. Moreover, the Black-throated Finch was once widespread. It is possible that sampled populations did not

experience significant decline until very recent years and genetic signatures of the population contraction are not yet evident in the sampled genetic markers.

Genetic isolation

Genetic analyses using microsatellite DNA in this study suggests that the current populations of the Black-throated Finch are substantially different, particularly between northern and southern forms. Subspecies genetic structure is also present in its congeneric Long-tailed Finch, for which the strong genetic separation occurs between regions due to a biogeographic barrier, the Ord Arid Intrusion in Western Australia (Rollins et al. 2012). However, the Black-throated Finch was historically distributed across a more or less continuous landscape to the northwest of the Burdekin Gap in northern Australia with both subspecies co-occurring north of Townsville. Hence, little evidence suggests that a geographical barrier has isolated the species across its range.

Populations isolated by habitat fragmentation could experience an overall reduction in genetic exchange between population patches, resulting in differentiation and sub-structuring of populations (Frankham et al. 2002). Recent landscape changes due to cattle grazing, agricultural clearing and open cut mining could have contributed to the contemporary differentiation of the Black-throated Finch populations. Such changes are less severe in Far North Queensland and Cape York Peninsula than other parts of Queensland. Birds in Townsville have been exposed to the most severe land clearing and habitat loss due to pastoralism, agricultural and urban developments. As a result, much habitats for the Black-throated Finch has disappeared for the Townsville population, hence more prominent genetic differentiation within the region comparing with other populations (as shown in our genetic structure analysis).

The haplotype network is poorly resolved due to a low level of mitochondrial DNA sequence polymorphism. All populations shared one haplotype (Hap_BA) with no distinct separation between populations, which is contrary to the genetic structure observed in microsatellite DNA. Discrepancies in patterns of population structure from mitochondrial and microsatellite DNA are not unusual (Johnson et al. 2003, Caparroz et al. 2009, Hefti-Gautschi et al. 2009, Wenzel et al. 2012). Where weaker mitochondrial structure is observed, female-biased dispersal is often present. Many bird species have female-biased dispersal and nuclear DNA based markers often have higher introgression rates in these species, e.g. *Ficedula* flycatchers (Saetre et al. 2003); *Hippolais* warblers (Secondi et al. 2003); and Amazonian manakins (Brumfield et al. 2001). Although no studies have yet examined the dispersal patterns of the Black-throated Finch, a high level of female-biased dispersal is unlikely in these birds, because field observations and banding data showed that both sexes have relatively small home ranges and the longest travel distance recorded was within 30km, suggesting a restricted dispersal (Forshaw et al. 2012).

Implications for conservation management

Currently, only the southern subspecies of the Black-throated Finch is recognised by the Australian Government as of conservation concern. However, I found that genetic diversity is similarly modest in both northern and southern subspecies. Although there is no direct evidence of reduced fitness in current populations, low genetic diversity can still decrease the capacity of the bird to cope with rapid environmental changes, increasing long-term extinction risk (Johnson et al. 2009, Frankham 2010b). Given the evidence that the genetic differentiation is weak, it is possible that quick management actions can restore habitat and population connectivity within regions with little genetic

downside. As a result, I suggest that conservation efforts should support continuous monitoring to identify any rapid population declines as land use intensification occurs throughout the species' range. Conservation management strategies that prioritise increased habitat suitability and restoration of habitat connectivity are encouraged.

CHAPTER V

Effects of landscape features on the fine-scale genetic structure of the endangered Black-throated Finch (*Poephila cincta cincta*) in northeastern Australia

(Photo credit: L. Stanley Tang)

ABSTRACT

Landscape features and heterogeneity are often reflected in a species' population genetic structure. In particular, habitat loss and fragmentation associated with landscape change often contribute to population decline and may result in changes to population structure and loss of genetic diversity of remaining populations. To understand the association between local landscape features and the population structure of a declining species, I examined the spatial genetic structure of 134 individuals of the threatened Black-throated Finch (Southern) (*Poephila cincta cincta*) in north-eastern Australia using 13 polymorphic microsatellite markers. I tested for isolation by distance and examined the relationship between the genetic differentiation and current landscape features by exploring the influences of landscape variables using ecological niche modelling (ENM) and least cost-path (LCP) analysis. I found strong spatial genetic structure among sampled individuals, shaped by an open water source (the Ross River Dam). ENM identified that elevation (41.1%), vegetation structure (21.3%) and water-related indices (28.1%) contributed the most to the distribution model. Geographic distance, and LCP cost distance were correlated to a lesser extent with the genetic distance. My results suggest that the current local landscape features and spatial separation have a combined effect on the fine scale genetic structure of the Black-throated Finch in the sampled area. Conservation management strategies that take into account habitat, water resources and maintaining dispersal corridors are likely to be effective for the persistence of the species in this region.

INTRODUCTION

Identifying relationships between heterogeneous landscapes and genetic structure

Lack of habitat connectivity can significantly impact wildlife populations by restricting dispersal and creating small isolated populations (Crooks and Sanjayan 2006). Species inhabiting landscapes fragmented due to human impacts often occur in small isolated populations, which can be more vulnerable to increased inbreeding depression, decreased genetic diversity and loss of adaptive potential (Keller and Waller 2002).

Small isolated populations are also more susceptible to demographic and environmental stochasticity (Hebblewhite et al. 2010). For example, patches of the breeding habitat (forests) of the endangered Golden-cheeked Warbler (*Dendroica chrysoparia*) were cleared for agricultural purposes in central Texas in the USA. Measures of genetic differentiation (genetic chord distance) were positively correlated with the percentage of agricultural lands, indicating that birds were not dispersing between patches of breeding habitats (Lindsay et al. 2008). Similarly, topographical differences between islands could be the major factor for shaping the strong genetic structure among populations of endangered species (e.g. Igawa et al. 2013). Water barriers can also reduce gene flow among mainland and island bird populations, providing evidence of the role of landscape features in generating genetic variations among populations (Wilson et al. 2011).

The relative importance and influence of landscape features on genetic structure can be evaluated by landscape genetic approaches. These approaches examine the interactions between environmental and evolutionary processes such as gene flow, genetic drift and selection (Manel and Holderegger 2013). The key distinction between landscape genetics and traditional population genetics is the inclusion of explicit tests of the

effects of spatial heterogeneity of landscapes on estimates of gene flow (Holderegger and Wagner 2008). One of the best approaches is to examine the correlation between genetic distance and cost distance (estimated from theoretical cost surfaces for movements between locations) to establish the effects of multiple environmental variables using geographic information systems (GIS) (Kierepka and Latch 2015). Many recent studies have demonstrated that this approach is a significant improvement over traditional methodologies (for example simple isolation-by-distance analysis) for explaining variation in genetic structure among populations. For example, incorporating landscape variables substantially increased the fit of the dispersal model (from an r^2 of 0.3 to 0.8) explaining genetic differentiation of the Blotched Tiger Salamander (*Ambystoma tigrinum melanostictum*) compared with the simple isolation-by-distance (IBD) approach (Spear et al. 2005). The genetic distance among populations of the Pacific Jumping Mouse (*Zapus trinotatus*) was better explained by movement distances along habitat pathways than using the simple Euclidean distance, demonstrating the power of considering landscape features in genetic structuring analysis (Vignieri 2005).

Contemporary landscape genetics usually analyses genetic differentiation within and among spatially separated groups. Sometimes, genetic populations or groups can be difficult to define, particularly when the species is distributed continuously over the landscape without clear population boundaries (Kierepka and Latch 2015). In these situations, clustering methods are usually used to define genetically distinct groups. However, patterns of spatial genetic structure such as isolation-by-distance can interfere with estimates of genetic clusters, producing inaccurate results (Latch et al. 2006). An alternative approach to avoid the difficulty in defining populations is to use individual-based analytical techniques, because this approach does not require a prior definition of populations. Instead, individual-based analysis group individuals based solely on the

genetic similarity and as a result can offer a means to investigate a wide range of fine-scale influences of ecological processes on gene flow. For example, an individual-based approach was used to assess the effects of landscape connectivity (the wooded habitat) on the dispersal of the European Roe Deer (*Capreolus capreolus*), because dispersing deer, especially yearlings, are not confined to discrete populations (Coulon et al. 2004). Abundance, effective population size and genetic differentiation of the Red-cockaded Woodpecker (*Picoides borealis*) were also linked to landscape heterogeneity (patch size and patch isolation) using an individual-based, spatially explicit population model which simulated population dynamics and the spatial genetic variation within heterogeneous landscapes (Bruggeman et al. 2010).

Impacts of changes in the landscape on the survival of granivorous birds in Australia

In Australia, habitat alteration and landscape changes since European Settlement have resulted in the decline of a number of amphibian, reptile, bird and mammal species, including the endangered Black-throated Finch (*Poephila cincta cincta*) (Morton 1990, Recher and Lim 1990). In particular, land modification for cattle grazing and agricultural development as well as changes in frequency and timing of fire have led to declines of granivorous birds in tropical and subtropical savannahs of northern Australia, despite the low density of human settlement (State of the Environment Advisory Council 1996, Franklin et al. 2005).

The movement patterns of the Black-throated Finch are poorly known. In the Townsville region, recent sighting records and research on the population around the Ross River Dam identified that the Black-throated Finch could move up to 20km (Rechetelo 2016). Other observations suggest that the bird may undertake some local

movements in response to changes in food availability as well as post-breeding dispersal (Baldwin 1976, Mitchell 1996, Natural Resource Assessment Environmental Consultants 2007). Therefore, it is likely that spatial genetic structures exist in populations of the Black-throated Finch and are associated with landscape features. Understanding such associations is important for the development and implementation of appropriate management strategies.

The Ross River Dam was constructed in 1971 for the purposes of water storage and flood mitigation in the Townsville region. The reservoir has a catchment area of 750km^2 and is now considered to be an ecologically important wetland area for Australia (Australian Nature Conservation Agency, 1996). Land use within the area has created a heterogeneous landscape that is ideal for spatial genetic analyses because the current landscape encompasses a wide range of features such as elevated areas (Mt Stuart to the north, Harvey Range to the west and Mt Elliot to east), cattle grazing areas, riparian zones and *Eucalyptus* woodlands.

In this chapter, I evaluate the influences of current landscape features on the spatial genetic structure of the Black-throated Finch. Specifically, I build a landscape resistance model based on selected habitat indicators and test this model against the spatial genetic structure using individual-based approaches within the population that persists around the Ross River Dam, south of Townsville in north-eastern Queensland, Australia. I hypothesise that: (1) the heterogeneous landscape in the sampling region has resulted in a distinct spatial genetic structure for the Black-throated Finch; (2) landscape features limiting the dispersal of the bird, such as presence of water, vegetation structure and topography are likely to be associated with patterns of the observed spatial genetic structure.

METHODS

Field sampling and genetic procedures

Study area and site selection

On the basis of sighting records in the past five years, I sampled at seven sites where the Black-throated Finch had been seen regularly around the Ross River Dam, in the Townsville region, Queensland, Australia (Table V-1). These sites were on the west and east sides of the dam. The north and south sides of the dam were occupied by urban developments and inaccessible private properties where birds were either not seen or impossible to sample. Distances between any two sites ranged from less than 1km to 18km (Figure V-1).

Table V-1 Sampling efforts of the Black-throated Finch (*Poephila cincta cincta*) in Townsville, Queensland Australia.

Sampling Sites	Directions of Ross River Dam	Sampling year	Sample size
CLW	West	2011-2013	23
LAP	West	2009*	48
DOD	Southeast	2012	8
FOD	East	2012-2013	41
MAD	East	2012	4
ANC	East	2011-2012	5
ARC	Northeast	2013	5
TOTAL			134

* Samples collected and provided by Kim Maute

Sample collection

I collected blood samples from each individual trapped using mist nets following strict protocols. I sampled a total of 86 individuals from seven sites around the Ross River Dam between 2011 and 2013. An additional 48 samples were obtained in 2009 from a previous study (Maute 2011). Details of the sampling procedure are in Chapter II.

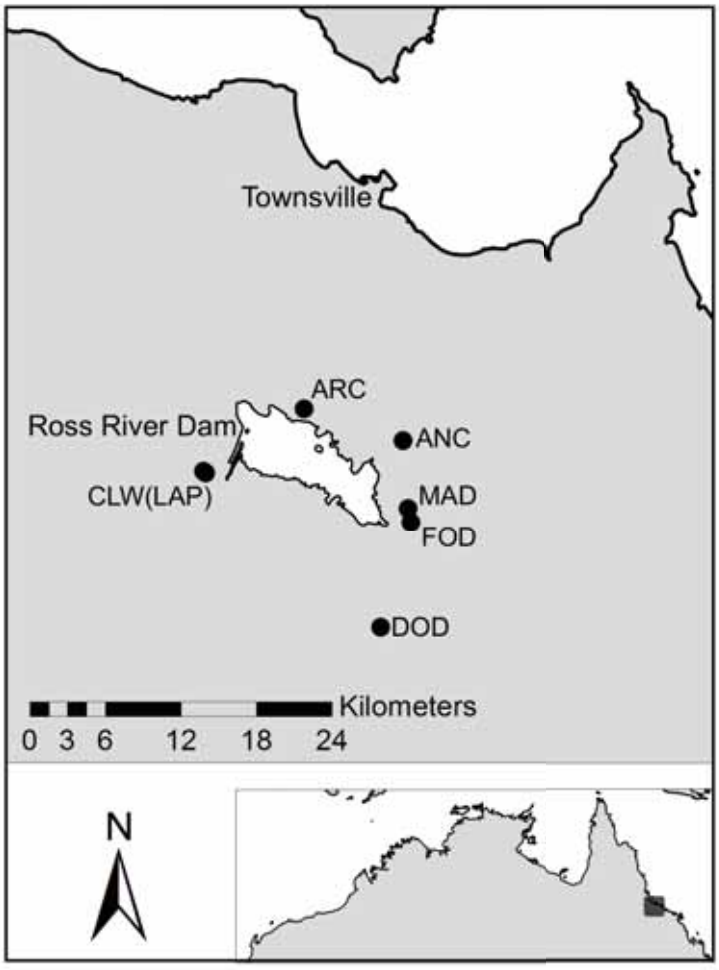

Figure V-1 Sampling locations (black dots) of the Black-throated Finch (*Poephila cincta cincta*) around the Ross River Dam in the Townsville region, Queensland

DNA extraction and Microsatellite genotyping

DNA was extracted from blood spots on FTA© cards and blood cells using ISOLATE Genomic DNA and ISOLATE II Blood DNA kits (Bioline, Australia), respectively. I modified the standard protocols recommended by the manufacturer to yield large amounts (typically between 5-10 μg) of purified DNA. Refer to Chapter II for details on the DNA extraction protocols. All individuals were genotyped at 18 microsatellite loci developed specifically for the Black-throated Finch (Tang et al. 2014). Details see Chapter II.

Statistical Analysis

Marker characteristics

Microsatellite genotype data were checked for evidence of typographic and scoring errors (large allele dropout, stuttering and the presence of null alleles) using MICROCHECKER version 2.2.3 (Van Oosterhout et al. 2004). I also performed the Ewens-Watterson test in POPGENE version 1.31 (Yeh and Boyle 1997) for neutrality of all microsatellite markers.

I checked all microsatellite loci for departures from Hardy-Weinberg equilibrium (HWE) by performing global χ^2 goodness-of-fit tests across population-specific F_{IS} (inbreeding coefficient) estimates and linkage disequilibrium (LD) between all pairs of loci in GENEPOP version 4.3 (Rousset 2008). I used Markov Chain Monte Carlo (MCMC) algorithms to estimate the probabilities of all tests with 10,000 dememorisation steps, 1,000 batches and 5,000 iterations per batch (Guo and Thompson 1992, Slatkin and Excoffier 1996). A sequential Bonferroni correction was also applied to all probability values of multiple comparisons to reduce Type I statistical error (Zar 1999).

Genetic structure

I analysed the population genetic structure and individual admixture based on microsatellite variation using the Bayesian clustering approach implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). This programme uses an MCMC algorithm to infer distinct genetic clusters (*k*), estimate allele frequencies in each cluster and population memberships of each individual. I chose the admixture model, assuming allele frequencies are correlated among populations, given the geographic extent of birds sampled in this study. Trial runs without locations as prior produced weak genetic

differentiation with no distinct clusters. Therefore, I used an individual-based approach with sampling locations as prior. The simulations were run for 10 replicates at each *k* value ranging from 1 to 10. I chose the upper limit of *k* to be greater than the number of sampled locations to account for any distinct genetic clusters that might be present within each population. Each replicate consisted of a burn-in period of 100,000 MCMC steps, followed by 2×10^5 iterations. Trial runs indicated the run durations were sufficient for likelihood values to stabilise. The best supported *k* values were determined by posterior probabilities (P(*D*)) and Δ*k* method (Evanno et al. 2005) in STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt 2012). The clusters output of the independent runs of the best supported *k* values were permuted and aligned using the "Full Search" algorithm to minimise the effects of "label switching" and "multimodality" in CLUMPP version 1.1.2 (Jakobsson and Rosenberg 2007). The output files were then visualised in DISTRUCT (Rosenberg 2004).

Landscape genetics

To investigate the influence of environmental variables on population genetic structure at a local scale, I employed environmental niche modelling (ENM) and the least cost path (LCP) analysis. ENM predicts the niche of a species using computer algorithms to represent its known distribution with habitat suitability values on a map with the integration of complex climatic and landscape variables (Elith and Leathwick 2009). The LCP is a function implemented in geographic information system (GIS) to determine the shortest path between geographical locations on the cost surface. It can be applied to map out likely dispersal corridors of a target species with appropriate movement cost assignment (Hirzel et al. 2006). By comparing genetic divergence among individuals between cost distances, I could test hypotheses on the effects of landscape features and other environmental variables on gene flow. Specifically, I

conducted the landscape genetic analysis in the following steps: (1) inference of expected habitat suitability from combinations of environmental variables (listed in the following paragraph) by ENM; (2) calculation of LCP distance matrix by inversing habitat suitability values; (3) analyses of correlations between the genetic distance, the Euclidean distance and the cost distance.

First, I performed ENM analysis in MAXENT version 3.3.3 (Phillips et al. 2006). I selected eight environmental variables that were reasonably expected to influence habitat suitability and movement of the Black-throated Finch: the regional ecosystems (RE), the ground cover disturbance index (GCDI), the normalised difference vegetation index (NDVI), the normalised difference water index (NDWI), the topographic wetness index (TWI), the normalised difference infrared index (NDII), slope and elevation. These variable layers were then trimmed to an area that encompassed all sampling locations (19.30-19.7171°S and 146.58-147.25°E) and resampled at the same spatial resolution of 0.0001 decimal degrees (~21m^2) because MAXENT requires the same resolution layers for ENM analysis. I used nearest neighbour assignment method for the resampling of RE and cubic convolution method for the rest of the variables.

The RE describes vegetation communities and structures that are consistently associated with a particular combination of topography within a bioregion (Sattler and Williams 1999). I used the original remnant regional ecosystems map (at a scale of 1: 100,000) from the Queensland Spatial Catalogue of Australia. Individual REs were categorised separately in the raster layer. The GCDI uses ground cover time series statistics, derived from the State-wide Landcover and Tree Study (SLATS) Landsat TM imagery over Queensland, to analyse the percentage ground cover within each regional ecosystem. This is used as an indication of the intensity of grazing and levels of disturbance. The

GCDI data package was acquired from the department of Environment and Heritage Protection, Queensland Government. I calibrated the corresponding GCDI within each RE as a grid raster for ENM analysis. The NDVI measures the greenness in an image and it is often used to monitor the overall biomass and to predict vegetation production (Lillesand et al. 2004). I prepared the NDVI layer within ArcMap version 10.2 using the raster calculator on Landsat 8 imagery provided by the US Geological Survey (USGS) Earth Explorer, based on the equation: $NDVI = 100 \times \frac{IR-R}{IR+R} + 100$, where *IR* is the pixel values from the infrared band and *R* is the pixel values from the red band (Lillesand et al. 2004). The NDWI uses green and near-infrared bands to visualise the presence of water bodies and the NDII measures the vegetation moisture condition. I prepared both the NDWI and NDII layers in the same manner as the NDVI, but with different equations: $NDWI = \frac{G-NIR}{G+NIR}$ (McFeeters 1996) and $NDII = \frac{NIR-SWIR}{NIR+SWIR}$ (Ji et al. 2011), where *G*, *NIR* and *SWIR* are the pixel values from the green, near-infrared and short-wave infrared bands respectively. The resolution of all bands acquired from Landsat 8 imagery is 30m. Elevation and slope data were compiled using the global digital elevation model (DEM) version 2 database (30 metre resolution) from the USGS Earth Explorer (Hjerdt et al. 2004). The TWI preparation followed the same manner as elevation and slope, using the equation: $\ln \frac{FA}{\tan b}$, where *FA* is the flow accumulation calculated by estimating water coverage on the DEM in ArcGIS version 10.2 (Wilson and Gallant 2000).

To construct the environmental niche model, a total of 44 unique occurrence records of the Black-throated Finch in the study area were used. The occurrence records of the Black-throated Finch within the study area between 2009 and 2014 included annual waterhole counts, targeted surveys as well as incidental sightings. Each individual

record was checked for accuracy and reliability based on the photographic evidence or confirmation by a member of the Black-throated Finch Recovery Team. Sightings that were reported multiple times at the same location were only counted as one record for the analysis.

I employed the leave-one-out jackknife approach to assess the statistical significance and to quantify measures of performance of ENMs using the default settings in MAXENT with 10 replicates. The jackknife test runs the model once with all variables, dropping out each variable in turn, and then with a single variable at a time (Peterson et al. 2007, Peterson et al. 2011). I reported the omission rate (OR) based on minimum training presence threshold and the averaged area under the curve (AUC) of the receiver operator characteristics (ROC) to measure the overall performance of the suitability model (Shcheglovitovaa and Andersona 2013). Values of the habitat suitability ranged from 0 (low) to 1 (high).

Second, I inverted the estimated suitability values to create a friction raster representing the cost of movement of the Black-throated Finch for each pixel on the map (Hirzel et al. 2006). I manually modified conspicuous barriers (the Ross River dam and urban built-up areas) to dispersal as high cost. Location-based pairwise LCP distances and corridors were estimated between all sampling locations in ArcGIS SDMtoobox version 1.1 (Brown 2014). I manually created the individual-based LCP distance matrix by assigning the same cost values to individuals that were collected from the same area.

Last, I examined the relationship between three different distance matrices (genetic, Euclidean and LCP cost distances between any two sampling locations) using Mantel tests. Specifically, I used the simple Mantel test to investigate the correlation between: (1) the genetic and the Euclidean distance matrices to detect possible patterns of IBD;

(2) the genetic and LCP cost distance matrices to detect possible patterns of isolation by environmental resistance; and (3) the LCP cost and Euclidean distance matrices to test whether these two variables are correlated. Individuals collected in the same sampling point were assigned using a random set of coordinates within a 20-metre radius of the sampling point. This is to ensure each sample has a set of unique GPS coordinates, and that does not fall into another grid cell on the map. The individual pairwise chord distance (D_c) was calculated in FSTAT version 2.9.3.2 (Goudet 1995) and used to construct the individual pairwise genetic distance matrix because D_c emphasises genetic drift over mutation, reflects population declines better than other indices, and thus may be particularly suitable for microsatellite data and fine-scale landscape genetic analysis (Kalinowski 2002). Then, I used the partial Mantel test to evaluate the correlation between the genetic and the LCP cost distance matrices alone after accounting for the possible effects of the IBD. Both Mantel and partial Mantel tests are implemented in PASSaGE version 2 (Rosenberg and Anderson 2011). Correlation significance was determined through 10,000 random permutations and Monte Carlo probability values were calculated.

RESULTS

Marker characteristics

There were no scoring errors associated with stuttering, large allele dropout or null alleles at any of the 18 microsatellite loci. Marker btfi43 and btfi36 were monomorphic and marker btfi23 contained 1base pair out-of-phase alleles. As a result, they were not used in further analysis. After Bonferroni corrections (corrected $\alpha = 0.0031$), H_O deviations from the HWE were detected at locus btfi27. As a result, it was excluded

from further analysis. Linkage disequilibrium of each pair of the remaining 13 loci was not detected within or among populations after Bonferroni corrections (corrected α = 0.0005).

Genetic structure

The individual based Bayesian clustering analysis in STRUCTURE and the maximum posterior probability ln P(*D*) analysis identified three distinct genetic clusters (*k* = 3, Figure V-2) with sampling locations as priori. Roughly, birds to the west of the Ross River dam belonged to one cluster (sites CLW and LAP), birds from the east of the dam comprised a second admixed cluster (sites FOD, MAD, ANC and ARC) and the remaining individuals from site DOD were well admixed.

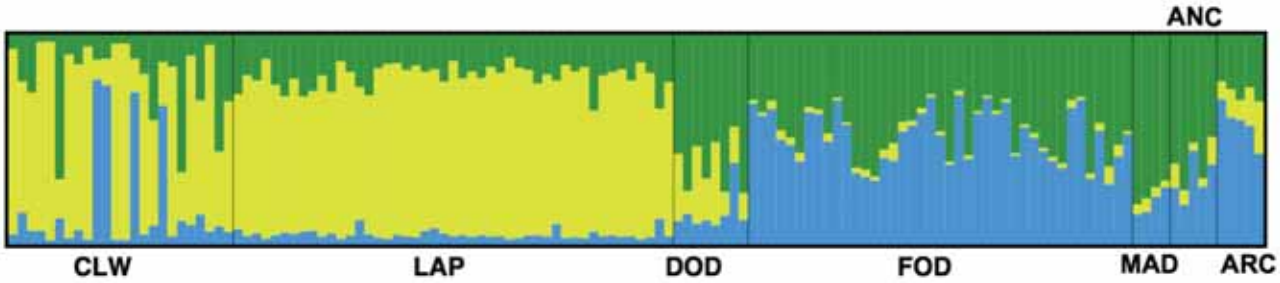

Figure V-2 Individual membership coefficients derived from Bayesian inference of the genetic structure of the Black-throated Finch population in the Townsville region, using admixture model (inferred genetic cluster *k* = 3) with sampling locations as prior. Each cluster is represented as a different colour and a single vertical line represents an individual

Landscape genetics

Of the 44 occurrence records, 38 were randomly selected by MAXENT for training to build the model and 6 were used to test the model. The minimum presence threshold is considered to be a conservative rule for determining whether an evaluation record falls into or out of the predicted area when calculating the omission rate (OR) (DeLong et al. 1988). The predicted model had an OR of 0 indicating the absence of over fitting. The

area under the curve (AUC) value of the model is 0.96 (±0.011 variance), also indicating a good fit to the species distribution. The estimated suitability map was congruent with known distribution areas of the Black-throated Finch (Figure V-3). Analysis of variable contributions showed that elevation, RE, NDWI and TWI contributed more than 80% to the overall model with elevation the highest (41.1%). The other three variables contributed much less to the model with GCDI contributing the least (0.1%) (Table V-2). The least-cost path (LCP) analysis identified potential dispersal pathways along west and east sides of the Ross River Dam between sampling locations (Figure V-4).

Table V-2 The relative contributions and jackknife test results of environmental variables to the MAXENT model. RE: regional ecosystems; GCDI: ground cover disturbance index; TWI: topographic wetness index; NDWI: normalised difference water index; NDVI: normalised difference vegetation index; NDII: normalised difference infrared index

Variables	Contribution	Train Gain[a]	Train Gain[b]	Test Gain[a]	Test Gain[b]	AUC[a]	AUC[b]
RE	21.28%	0.520	1.169	0.554	1.767	0.792	0.971
GCDI	0.109%	0.003	1.236	0.007	1.642	0.520	0.953
TWI	10.76%	0.246	1.181	0.479	1.480	0.831	0.945
NDWI	17.33%	0.274	1.241	0.064	1.727	0.551	0.961
NDVI	1.927%	0.306	1.205	0.164	1.631	0.632	0.952
NDII	6.775%	0.210	1.099	0.323	1.515	0.670	0.939
Elevation	41.07%	0.778	0.857	1.198	0.962	0.916	0.871
Slope	0.762%	0.197	1.235	0.112	1.711	0.621	0.960

a jackknife test run with only the selected variable
b jackknife test run without the selected variable

In both simple Mantel tests, the genetic distance matrix was significantly, but weakly correlated with Euclidean (Table V-3) and LCP cost distance matrices ($R = 0.081$, $P < 0.001$ and $R = 0.083$, $P < 0.001$, respectively). Both Euclidean and LCP cost distance matrices explained less than 1% of the total variation in the genetic distance ($R^2 = 0.0065$ and 0.0067 respectively). Because Euclidean and LCP distances were strongly correlated ($R = 0.982$, $P = 0.001$), I used the partial Mantel test to control the effect of

IBD. By keeping Euclidean distance matrix constant, the LCP distance matrix did not have a significant correlation with the genetic distance (R = -0.003, P = 0.475).

Table V-3 The Euclidean distance (in km, above the diagonal) and the least cost path (LCP) distance (below the diagonal) between each pair of sampling locations

	CLW	LAP	DOD	FOD	MAD	ANC	ARC
CLW		0.073	18.008	15.919	15.469	15.039	8.984
LAP	0.001		18.054	15.987	15.538	15.112	9.048
DOD	0.216	0.216		8.642	9.674	14.947	18.312
FOD	0.204	0.204	0.095		1.135	6.541	12.086
MAD	0.202	0.202	0.106	0.011		5.413	11.125
ANC	0.253	0.253	0.166	0.066	0.055		7.837
ARC	0.130	0.130	0.240	0.144	0.133	0.086	

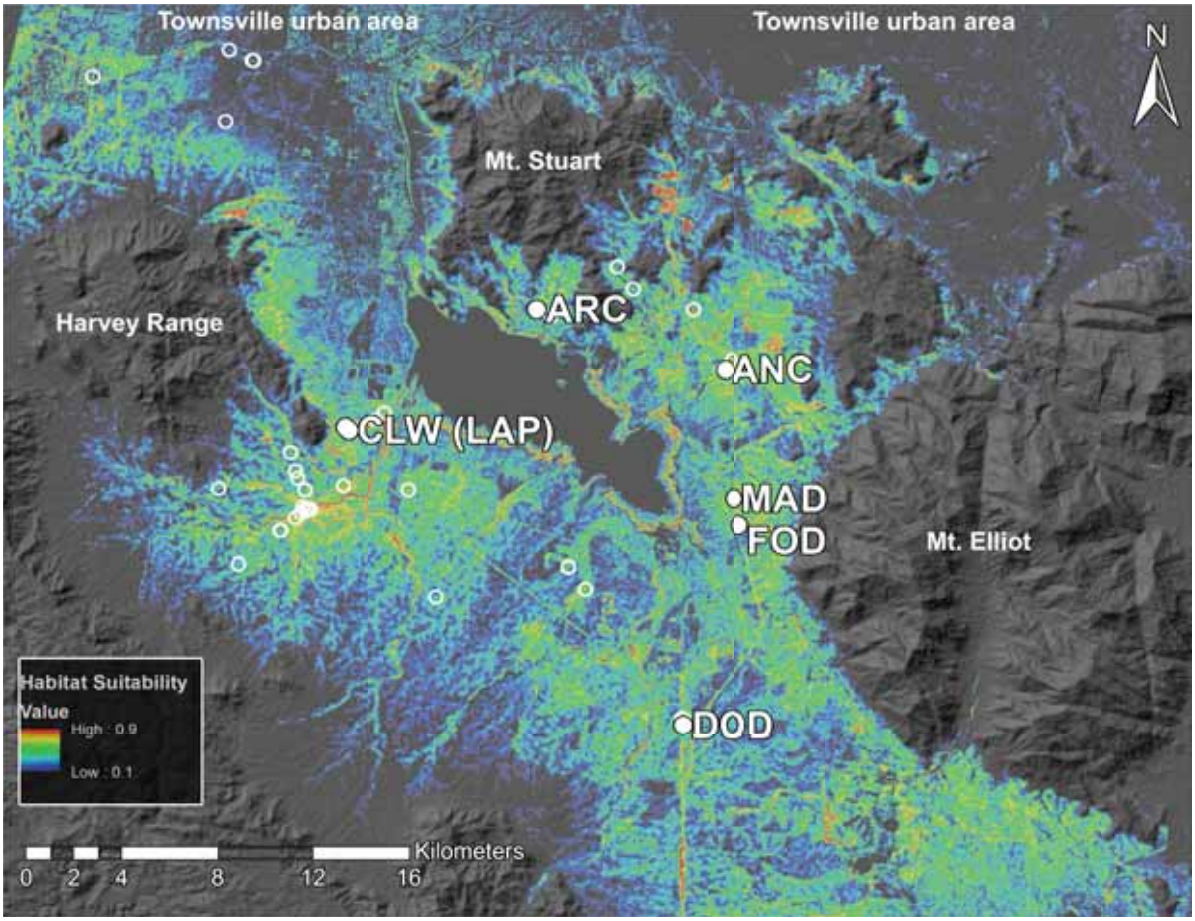

Figure V-3 Habitat suitability of the Black-throated Finch around the Ross River Dam in Townsville, Queensland, Australia. Clear white circles represent sighting records between 2009 and 2014. White dots with black outlines show sampling locations of this study

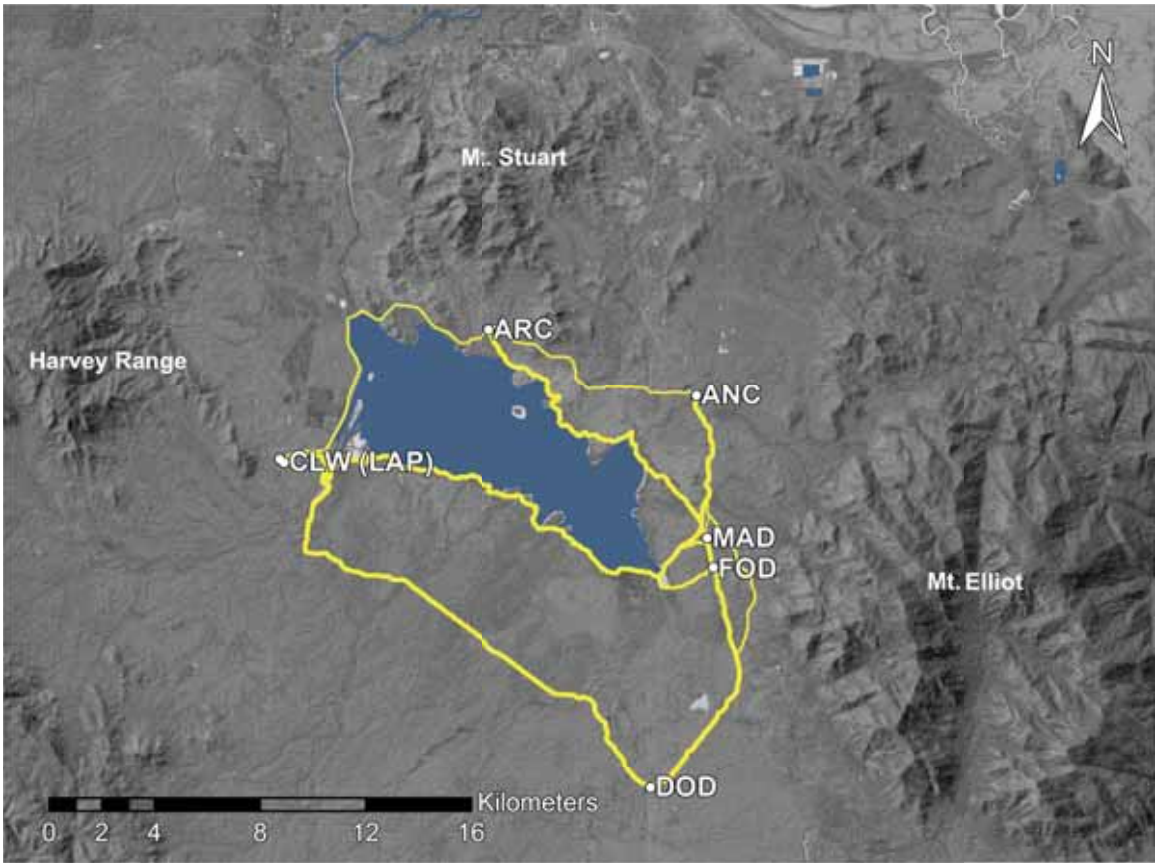

Figure V-4 Identified least-cost pathways (yellow lines) from PATHMATRIX simulations, representing potential dispersal corridors of the Black-throated Finch among sampling locations (white dots) in the Townsville region. Thickness indicates the cost values – the thicker the line, the lower the cost of dispersal

DISCUSSION

To examine the influence of local landscape features on the genetic structure of the threatened Black-throated Finch, I first analysed the population genetic structure of more than 100 individuals from seven locations around the Ross River Dam in the Townsville region. Then, the environmental niche modelling (ENM) and the least cost path (LCP) analysis were used to to estimate the habitat suitability. My results provided evidence that large bodies of open water, i.e. the Ross River Dam, reduced gene flow among individuals within the area. I also demonstrated landscape variables, such as the vegetation structure are potential factors driving the fine-scale (within 20km) genetic

structure of the Black-throated Finch population around the Ross River Dam. This work provides essential information for the identification of potential habitat barriers for the bird. It also aids in developing appropriate habitat management strategies to maintain high levels of genetic connectivity of the Black-throated Finch.

Spatial genetic structure and isolation by distance (IBD)

The Bayesian clustering analysis based on microsatellite markers revealed varied levels of genetic separation between groups of birds sampled on west and east sides of the Ross River Dam that are less than 20km apart. Field observations and banding records show that the Black-throated Finch in the area rarely move further than 18km. This means that the population is more likely to be isolated as a result of habitat fragmentation. This demonstrates that distinct spatial population structuring within the Black-throated Finch population around the Ross River Dam in the Townsville region can occur at a scale of 10-20km.

Birds may be expected to experience lower levels of landscape resistance because the ability to fly allows them to cross potential geographical gaps more rapidly and to cover larger distances more efficiently than other terrestrial vertebrates. However, population genetic structures at fine spatial scales have been detected. For example, significant correlations between genetic and geographic distances were detected within populations of the Song Sparrow at distance classes less than 10km in North America (Wilson et al. 2011). Similarly, fine spatial scale (less than 200m) genetic structures were found in other passerines such as the White-breasted Thrasher (*Ramphocinclus brachyurus*) (Temple et al. 2006) and the Superb Fairywren (*Malurus cyaneus*) (Double et al. 2005).

Water barriers have been shown to limit movement in many avian taxa over short distances. For example, the lower portion of the Amazon River is wider and more open; hence it is a more effective barrier to dispersal and gene flow than other parts of the river for many forest-restricted species, particularly the antbirds (Thamnophilidae) (Hayes and Sewlal 2004). It is evident that the presence of the dam acted as a barrier to gene flow within the population of the Black-throated Finch around the Ross River Dam near Townsville. Field observations suggest that the Black-throated Finch prefers to drink water from waterholes, small farm dams surrounded by trees and farm troughs or water tanks (Immelmann 1982, Natural Resource Assessment Environmental Consultants 2007, Forshaw et al. 2012). Large bodies of water (e.g. the Ross River Dam) are less frequently visited by the bird probably due to the high risk of predation or to the high energy cost of crossing over as demonstrated in many small passerines (e.g. Brawn et al. 1996, Adams and Burg 2015).

Although weak, the IBD structuring found in our analysis suggests that the geographical distance that the Ross River Dam created also restricts the gene flow of the Black-throated Finch within the area. Variable IBD patterns can be observed across a species' range depending on the spatial scale examined (Hutchison and Templeton 1999, Castric and Bernatchez 2003, Garnier et al. 2004). Studies have suggested that many factors, including localised differences in migration, time since populations have been present in a region and local landscape heterogeneity can influence IBD patterns (Coulon et al. 2004, Bradbury and Bentzen 2007). The Ross River Dam was constructed in 1971 for the purposes of flood mitigation and water storage for the Townsville region. Within the 45 years since the dam was constructed, the current genetic differentiation within the population developed and clustering analyses and shows it was associated with the geographical distance created by the dam. However, it is possible that such a level of

differentiation is not significant enough to support a strong IBD pattern. The weak IBD pattern may also be because the spatial intervals between sampled locations were relatively far apart and a finer gradient of distances between sampling locations is needed for a stronger IBD pattern (Smouse and Peakall 1999). Finally, other local landscape features such as mountain ranges, vegetation structure and presence of small water bodies within the region also had a stronger influence than spatial distance in the study area (as discussed below).

Influences of landscape variables

Based on the jackknife test of variable importance, I identified that elevation and vegetation structure (RE) are important contributors to the distribution of the Black-throated Finch in the Townsville area. Water related indices (NDWI and TWI) also contributed significantly to the distribution model of the Black-throated Finch. Strong contributions from both RE and water-related indices are also consistent with the results from field surveys and previous studies that evaluated habitat requirements of Australian grassfinches (Jennings and Edwards 2005, Maute 2011, Forshaw et al. 2012). Their results showed that the Black-throated Finch is often found in the vicinity of water, and mainly associated with specific vegetation structure such as grassy, open woodlands and forests (Black-throated Finch Recovery Team 2007).

The association between the distribution of Black-throated Finch and the landscape variables (identified above) around the Ross River could be the result of balanced energy intake and cost of foraging and breeding. The Black-throated Finch prefers to forage on open ground (e.g. roadsides, and small clearings in the understorey) surrounded by trees or shrubs to which they can fly when disturbed or alarmed (Immelmann 1982, Natural Resource Assessment Environmental Consultants 2007,

Rechetelo 2016). Such foraging behaviour suggests that the bird requires a clear line of sight to detect both aerial and ground predators. Dense ground cover with tall vegetation or elevated ground surface may reduce the ability to detect danger; hence increase the cost of foraging (Tang and Schwarzkopf 2013). The daily requirement of water also determines that the Black-throated Finch inhabits areas in the vicinity of water bodies, e.g. waterholes, small dams, creeks and water troughs. Dry and elevated areas, such as Mount Stuart (north of the Ross River Dam) and Harvey Range (west of the Ross River Dam), have higher evaporation rates and faster runoff due to shallow soils and exposed grounds (McCain 2009). As a result, the water availability is much lower than it is at lower altitudes.

Mantel tests revealed that the observed genetic distance matrix was weakly correlated with the LCP distance matrix. Although less than 1% of the variation in genetic distances could be explained by the LCP cost distance calculated from the ENM, I detected a significant association between landscape features and the genetic structure at a spatial scale of less than 20km. However, the relationship disappeared when the geographic structure common to both matrices was accounted for (partial Mantel R = -0.003, P = 0.475). The strong correlation between the LCP cost and geographic distances showed that the explanatory power of the LCP cost is probably due to the spatial patterns of the LCP cost distance. Therefore, landscape features alone do not explain the spatial genetic structure of the Black-throated Finch in the Townsville region.

Recent geographical fragmentation within a population may not necessarily result in a significant genetic differentiation between sub-populations, because the allele frequency differences may take a long time to build up and become fixed. For example, it would

take 2.77N_e (effective population size) generations for an allele with an initial frequency of 0.5 to become fixed (Kimura and Ohta 1969). My Bayesian clustering analysis based on the admixture model showed that many individuals within each cluster had mixed origins indicating some level of gene flow still exists across the Ross River Dam in the Townsville region. This is also supported by the LCP analysis, in which I identified multiple possible dispersal corridors (Figure V-3) for gene flow. Substantial local landscape changes occurred only 30 – 50 years ago, a weak association between the genetic structure and other landscape features is expected. Nonetheless, by sampling individuals from seven sites that span 0.07-18km around the Ross River Dam in the Townsville region, I was able to show that the combination of environmental and geographical factors (such as landscape features, the presence of Ross River Dam, and the geographical distance) is likely to reflect in the spatial genetic structure observed.

Implications for conservation management

The inferences associated with the fine-scale spatial genetic structure have significance for the conservation of the Black-throated Finch. Understanding the scale at which gene flow predominates can indicate the approximate scale of demographic independence (e.g. Diniz and Telles 2002).

Dispersal over small spatial scales is important to maintain genetic connectivity (Sunnucks and Taylor 2008) and this work highlights the relationship between landscape features (such as open water, mountain ranges, long geographic distance, unsuitable vegetation structure and absence of small water bodies) and dispersal limitation in the Black-throated Finch. Although a strong spatial genetic structure was detected, there was some level of gene flow between sub-populations around the Ross River Dam. Therefore, managing the Townsville population of the Black-throated Finch

as a whole with conservation strategies focusing on retention of corridors and suitable habitat would be a priority to ensure species persistence in the region. It is also important to conduct on-going genetic monitoring to maintain genetic diversity of the population.

CHAPTER VI

Conservation values and genetic diversity retention from wild and captive populations of a threatened species, the Black-throated Finch (*Poephila cincta*)

(Photo credit: L. Stanley Tang)

ABSTRACT

Ex situ conservation programmes and reintroductions of captive bred animals are a frequent component of endangered species conservation strategies. However, captive populations may go through repeated bottlenecks, leading to a lack of genetic diversity and to genetic differentiation between various captive and wild populations. In this chapter, I examine the genetic diversity of 96 captive individuals of the Black-throated Finch acquired from various aviculturists in Australia, using 14 polymorphic microsatellite markers. I also compare the genetic diversity (measured as heterozygosity, H_O and allele richness, *r*), effective population size (N_e), inbreeding (*F*) and relatedness (R_r) between captive and wild populations. I find that all captive populations have a lower level of genetic diversity (average H_O = 0.35 among captive populations versus average H_O = 0.45 in the wild; average *r* = 2.34 in captivity versus 3.08 in the wild); smaller effective population sizes compared with all wild populations; higher levels of inbreeding (*F* = 0.114 in the wild versus *F* = 0.216 in captivity) and similar levels of individual relatedness. Individual assignment tests identify mixed origins of captive birds, the majority of which cannot be traced back to current wild populations that were sampled in this project. My results suggest that the Black-throated Finch in captivity has lost genetic variability to some degree and increased levels of inbreeding may potentially reduce its viability. However, the presence of captive birds that originated from wild populations that are now believed to be extinct makes these individuals particularly important. These findings allow us to recommend that *in situ* conservation strategies should be the priority and if captive breeding programs are considered, birds of known origins should be kept and bred separately.

INTRODUCTION

In situ and ex situ conservation

In situ and *ex situ* strategies are distinct approaches for the conservation of wildlife (Pritchard et al. 2011). *In situ* conservation emphasises the protection of threatened species in their natural habitats, with strategies usually focused on the improvement or protection of suitable habitats and ecosystems through the establishment of national parks, nature reserves and other conservation areas, as well as the removal of threatening processes (Pritchard et al. 2011). *Ex situ* conservation, on the other hand, focuses on the preservation of endangered species in human controlled environments, such as zoos, aquaria, botanical gardens, seed banks and captive animal breeding centres. Such strategies are usually the last resort to protect an endangered species because of the organism's inability to survive in the wild without human intervention (Witzenberger and Hochkirch 2011).

In situ conservation preserves biodiversity, which includes both declining species and the evolutionary processes that enable them to adapt to the changing environment. Such evolutionary processes enable species to develop potentially important and useful genetic traits in response to environmental stochasticity (Frankham et al. 2002). Although *in situ* conservation represents the preferred way and arguably the most effective means of conserving threatened and endangered species, viable populations of some species can only be maintained *ex situ* (Pelletier et al. 2009). For example, the endangered Asian Crested Ibis (*Nipponia nippon*) persisted as only two pairs rediscovered in 1981. Without captive breeding, the species would have an extremely high probability of extinction due to the loss of genetic diversity and elevated vulnerability to environmental catastrophes (Zhang et al. 2004). Currently, more than 60

species are found only in captivity (animals) or under cultivation (plants) (BirdLife International 2012) and about 3,000 species are likely to require *ex situ* breeding to prevent extinction in the next 200 years (Seal 1991, Magin et al. 1994).

Despite the increasing demand for *ex situ* conservation programmes, a number of challenges must be overcome in order to increase the likelihood of survival of captive populations and the success of reintroduction programmes. First, it is important to minimise the inbreeding depression and the accumulation of deleterious alleles. Captive populations of many species originate from a small number of founders as a result of difficulties in field collections or simply because there are no more in the wild (Leberg and Firmin 2008). For example, the last wild California condor (*Gymnogyps californianus*) was brought into captivity in 1987 and the surviving population to date are all descendants of the last 14 wild birds. The severe population bottleneck has led to an increased frequency of a lethal allele for chondrodystrophy (Ralls and Ballou 2004). Second, genetically distinct populations in the wild should ideally be represented in the captive population to maximise genetic variability, and so that the ability to adapt to different conditions can be retained. This is particularly important for species that are patchily distributed and/or show strong genetic structuring among existing populations. For example, founders of the captive Jamaican Yellow Boa (*Epicrates subflavus*) originated from one wild population even though more wild populations exist. Decreased genetic variability was clearly identified by the fact that the captive population had fewer alleles compared with the wild populations (Tzika et al. 2009). Furthermore, the genetic adaptation to captivity should be minimised, because captive bred individuals frequently have reduced fitness when reintroduced to the wild resulting in lowered reintroduction success of captive-bred individuals compared with translocations of wild individuals (Williams and Hoffman 2009).

In situ conservation is considered to be the legal and institutional priority for many conservation organisations and agreements, such as the Convention on Biological Diversity (Pritchard et al. 2011). *Ex situ* strategies can however be a useful complement to *in situ* measures, because they directly preserve the target species against extinction and provide valuable resources which may aid in the recovery of endangered species. However, many *ex situ* populations are not established until the species has gone through major declines and there are few surviving individuals in the wild (Wilson et al. 2012). Consequently, there is limited retention of original genetic diversity. The International Union for Conservation of Nature (IUCN) recommends that a detailed review of the species should be carried out before *ex situ* conservation programmes are established. Such reviews ideally include all factors related to the life history, taxonomy, current population status, demographic and genetic viability as well as current threats to species persistence (IUCN/SSC 2014).

Conservation of the Black-throated Finch

In Australia, habitat alteration and landscape changes have resulted in the decline of many faunal groups, including granivorous birds, and appropriate conservation management strategies are needed (Lindenmayer 2009). In particular, the tropical and subtropical savannahs of northern Australia, which support the largest granivorous bird assemblages, have experienced the greatest declines (State of the Environment Advisory Council 1996, Franklin et al. 2005).

The Black-throated Finch (*Poephila cincta*) in particular has suffered a substantial decline as a result of habitat modification and loss. The current conservation efforts are *in situ*, such as the acquisition of population information (e.g. population size, structure and abundance), understanding the ecology and identifying the relative importance of

key threats (Mitchell 1996, Black-throated Finch Recovery Team 2007, Maute 2011, Rechetelo 2016).

The Black-throated Finch is generally common in Australian aviculture. However, it is less popular than other related grassfinches such as the Long-tailed Finch (*Poephila acuticauda*) due to certain behavioural traits (such as nest destructiveness and aggressiveness towards other finches) and colour variations (Forshaw et al. 2012). The total number of the Black-throated Finch held by aviculturists in Australia was estimated to be approximately 593 in 2011 (Fitt and Pace 2011).

Colour mutations and hybridisation with other finch species have also been observed among aviary birds. Roughly 15% of the total number of the captive Black-throated Finch are identified as colour mutants (Fitt and Pace 2011). The high mutation rate among captive birds raises concerns for the future viability of the species in captivity, and may also indicate the presence of potential negative genetic processes such as bottlenecks and inbreeding.

To evaluate the potential value of captive populations in species persistence for a threatened granivorous bird, I assessed the potential genetic conservation values of the Black-throated Finch held by aviculturists in Australia. In particular, I compare the genetic diversity of groups of captive and wild populations of birds; estimate the effective population sizes; and test captive birds for genetic bottleneck, relatedness and inbreeding depression. I hypothesise that (1) through the process of domestication, birds in captivity have lowered genetic diversity compared with those in the wild; (2) signatures of genetic bottlenecks are present as a result of high levels of inbreeding in captivity; (3) individuals in captivity are more related than those in the wild.

METHODS

Field sampling and genetic procedures

Sample collection

I sampled wild birds from four locations across the current range where the Black-throated Finch had been seen regularly since 2009. The southern form (*P. c. cincta*) was sampled in the Townsville region as well as in the Desert Uplands in central Queensland. The northern form (*P. c. atropygialis*) was sampled at Mareeba in far north Queensland. I collected small amounts (30-50μL) of blood from each individual trapped using mist nets following strict protocols. Blood samples were applied to FTA© card and dried for further analysis. Samples for Lakefield National Park on Cape York Peninsula were obtained from a previous study (Maute 2011) and stored as blood cells in 70% ethanol. All wild samples were collected between 2009 and 2013. Detailed sampling protocols are in Chapter II.

I sourced samples of captive birds from Australian aviculturists, who keep records of each individual bird they possess, in the states of Queensland and New South Wales. I only assigned the location information to those birds, of which owners had records of the origin. Birds that had no origin information were included in one group (see Table II-1 in Chapter II). Each finch owner collected blood samples from their own birds following the same FTA© card protocol as mentioned above.

DNA extraction and microsatellite genotyping

DNA was extracted from blood spots on FTA© cards and blood cells using ISOLATE Genomic DNA and ISOLATE II Blood DNA kits (Bioline, Australia), respectively. I modified standard protocols as recommended by the manufacturer to yield large

amounts (5-10μg) of purified DNA. Refer to Chapter II for details on the DNA extraction protocols. All individuals were genotyped at 18 polymorphic microsatellite loci developed specifically for the Black-throated Finch (Tang et al. 2014). Details see Chapter II.

Statistical Analysis

Marker characteristics

All microsatellite genotype data were checked for evidence of typographic and scoring errors (large allele dropout, stuttering and the presence of null alleles) for both wild and captive populations of the Black-throated Finch using MICROCHECKER version 2.2.3 (Van Oosterhout et al. 2004).

I analysed all microsatellite loci to test for departures from Hardy-Weinberg equilibria (HWE) within each population by performing global χ^2 goodness-of-fit tests across population-specific F_{IS} (inbreeding coefficient) estimates, and linkage disequilibrium (LD) between all pairs of loci in GENEPOP version 4.3 (Rousset 2008). I used Markov Chain Monte Carlo (MCMC) algorithms to estimate the probabilities of all tests with 10,000 dememorisation steps, 1,000 batches and 5,000 iterations per batch (Guo and Thompson 1992, Slatkin and Excoffier 1996). A sequential Bonferroni correction was also applied to all probability values of multiple comparisons to reduce Type I statistical error (Zar 1999).

Genetic variability

In order to compare the genetic diversity between wild and captive groups of the Black-throated Finch, I calculated the observed heterozygosity (H_O), the unbiased expected heterozygosity (H_E) and the number of private alleles (N_{PA}) using GenAlEx version 6.5

(Peakall and Smouse 2006, 2012). The average allelic richness (number of alleles, *r*) and allele frequencies were calculated in FSTAT version 2.9.3.2 (Goudet 1995). The method used to calculate *r* incorporates a rarefaction method to compensate for unequal sample sizes (el Mousadik and Petit 1996), as is appropriate given the sampling design employed. For captive birds, I treated individuals that were of the same origin as a single population. There were only two individuals from NSW, insufficient to form a separate population. I grouped these two individuals with SEQ because SEQ is geographically adjacent to NSW. I also compared the genetic differentiation between each pair of populations using the unbiased Nei's genetic distance (*D*) and tested the significance of population differentiation by analyses of molecular variance (AMOVA) using allele frequencies in ARLEQUIN version 3.5.1.3 (Excoffier and Lischer 2010). I used all captive populations as one group and all wild populations as a second group so that the differentiation among and within populations and groups could be compared. The significance of the analysis was tested running 10,000 permutations.

I used two standard *ad hoc* methods to test for signatures of recent genetic bottleneck within the captive population. First, heterozygosity excess was tested assuming a two-phase mutation (TPM) model with 95% stepwise mutations, 5% multiple-step mutations, and a variance among multiple steps of 12, in BOTTLENECK 1.2.02 (Piry et al. 1999). The TPM model is considered to be the most appropriate for microsatellite data (Piry et al. 1999). Significance of heterozygosity excess over all loci was determined with a one-tailed Wilcoxon sign rank test after 10,000 iterations as implemented in the program. Second, I used the *M*-ratio method developed by Garza and Williamson (2001) to calculate the ratio (*M*) of the total number of alleles to the range in allele sizes, and its critical value M_C (5% of values fall below M_C as determined by simulations). *M* and M_C were estimated using M_P_VAL and

CRITICAL_M, respectively (Garza and Williamson 2001). I assigned three required parameters that were required for both programmes with recommended values: (1) pre-bottleneck $\theta = 10$; (2) the percentage of one step mutations, $\rho_s = 0.9$; and (3) the mean size of non one-step mutations, $\Delta_g = 3.5$. Each set of simulations consisted of 10,000 iterations.

Effective population sizes

To measure the ability of populations to maintain genetic variation and to characterise the risk status, I estimated the effective population sizes (N_e) for all wild and captive populations using a combination of three methodologies. This is to increase the reliability of the estimation and is due to the fact that N_e estimates vary depending on the influencing factors that each method is based on (Waples and Do 2010). First, I estimated N_e based on linkage disequilibrium taking into account the effects of genetic drift on allele frequencies (Hill 1981) with bias correction (Waples and Do 2010). Second, I calculated N_e from molecular coancestry, considering the level of allele sharing within each population (Nomura 2008). Both linkage disequilibrium and molecular coancestry methods are implemented in NeEstimator version 2.01 (Peel et al. 2004). Last, I used a Bayesian approach, assuming a continuous Brownian motion model for microsatellite data, to estimate the mutation-scaled effective population size $\Theta = 4N_e\mu$ (where μ is the mutation rate per site per generation, assumed constant for all loci) implemented in MIGRATE-n version 3.6.8 (Beerli 2009). The Bayesian inference in this programme accounts for the influence of gene flow on the within-population genetic diversity (Beerli and Felsenstein 2001).

Genetic structure

I compared the population genetic structure and individual admixture between wild and captive populations based on microsatellite variation using the Bayesian clustering approach implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). This programme uses an MCMC algorithm to infer distinct genetic clusters (*k*), estimate allele frequencies in each cluster and population memberships of each individual. I chose an admixture model, assuming allele frequencies are correlated among populations. First, I used the sampling locations as prior for wild populations and considered all captive individuals as one population. Second, I assessed the population structure of the captive birds separately using their origins as prior. Birds that were of unknown origins were grouped together. Trial runs without locations as prior produced weak genetic differentiation with no distinct clusters. The simulations were run for 10 replicates at each *k* value ranging from 1 to 10. I chose the upper limit of *k* to be greater than the number of assigned populations to account for any sub-structuring. Each replicate consisted of a burn-in period of 100,000 MCMC steps, followed by 2×10^5 iterations. Trial runs indicated the run durations were sufficient for likelihood values to stabilise.

The best supported *k* values were determined by posterior probabilities (P(*D*)) and Δk method (Evanno et al. 2005) in STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt 2012). The clusters output of the independent runs of the best supported *k* values were permuted and aligned using the "Greedy Search" algorithm to minimise the effects of "label switching" and "multimodality" in CLUMPP version 1.1.2 (Jakobsson and Rosenberg 2007). Outputs were then visualised as a cluster plot in DISTRUCT (Rosenberg 2004).

I also performed assignment tests in GenAlEx to genetically assign individuals of the captive population to their population origin in the wild. For captive birds of known origin, I compare the assignment results with the reported origins so the extent of genetic exchange within a captive population can be identified. For individuals of unknown origin, I identified the most likely source populations. I used the "leave one out" option in the software to account for potential bias. Specifically, this method removes the genotype of the test individual from the population it was sampled in before estimating the expected allele frequencies (Efron 1983).

Genetic relatedness and inbreeding

In order to estimate the genetic similarity between each pair of individuals in both wild and captive populations, I calculated the pairwise relatedness values based on a maximum likelihood method that uses the genotypes of a triad of individuals (trioML). This method reduces the chance of misidentifying genes identical in state as identical-by-descent and allows for inbreeding as well as genotype errors in data (Wang 2007). The simulated mean relatedness values were then compared to the theoretical relationship classification (0.5 for parent-offspring and full siblings, 0.25 for half siblings and 0 for unrelated) and 95% and 99% confidence intervals were obtained by bootstrapping with 1,000 repeats. I then estimated the individual inbreeding coefficient using the same statistical approach. Both analyses were implemented in COANCESTRY version 1.0.1.5 (Wang 2010). Last, I compared the averaged relatedness and inbreeding coefficient between wild and captive populations in COANCESTRY using the same bootstrapping procedure to estimate the confidence intervals. All captive populations were combined as a single group for both relatedness and inbreeding analyses to increase the sampling size and also because many captive

birds are known to be exchanged between breeders and pooling them is appropriate for identifying potential genetic relatedness between individuals.

RESULTS

Marker characteristics

There was no evidence for scoring errors associated with stuttering, large allele dropout or null alleles at any of the 18 microsatellite loci scored. Neutrality tests confirmed that all of the microsatellite and mitochondrial DNA markers were neutral. Markers btfi43 and btfi36 were monomorphic and marker btfi23 contained 1base pair of out-of-phase alleles. Therefore, they were not used in further analysis. After Bonferroni corrections (corrected $\alpha = 0.0031$), H_O deviations from the Hardy-Weinberg Equilibrium (HWE) were detected in at least one population at 5 loci (btfi04, btfi27, btfi31-2, btfi40 and btfi41). However, only locus btfi27 showed consistent deviation across all populations and was excluded from further analysis. Linkage disequilibria of each pair of the remaining 14 loci were not detected within or among populations after Bonferroni corrections (corrected $\alpha = 0.0005$). This set of markers showed a modest level of polymorphism over both wild and captive populations of the Black-throated Finch. The number of alleles ranged from 2 (btfi36) to 14 (btfi05) with an average of 4.5 (± 0.365 standard error) alleles per locus.

Genetic diversity and structure

The number of private alleles was highest in the TSV population ($N_{PA} = 4$), followed by the CEQ population ($N_{PA} = 3$). Apart from FNQ, all wild populations had private alleles. Among the captive populations, private alleles were present only in SEQ ($N_{PA} = 2$) and

UNK (N_{PA} = 1). The lowest observed heterozygosity was also detected in one of the captive populations (AYR) with an average of 0.274 (± 0.057 standard error). The averaged observed heterozygosity among wild populations was significantly higher than it was among captive populations (P = 0.027 < 0.05, 1,000 permutations). The allele richness showed a moderate level of variation within each population (ranged from 2.07 to 3.24). The averaged r among captive populations was significantly smaller than that among wild populations (P = 0.022 < 0.05, 1,000 permutations) (Table VI-1).

Table VI-1 Measurements of genetic diversity and results of genetic bottleneck analysis for each of the Black-throated Finch (*Poephila cincta*) populations. N_{PA}: number of private alleles; H_O: observed heterozygosity; H_E: expected heterozygosity; r: average allelic richness. M: ratio of total number of alleles to the range of allele sizes; M_C: critical value of M; P_W: probability value of one-tailed Wilcoxon sign rank test for heterozygosity excess. For M-ratio method, if $M < M_C$; and for Heterozygosity excess method, if $P_W < 0.05$, a recent bottleneck is likely to be present

Region	N_{PA}	H_E **(SE)**	H_O **(SE)**	r **(SE)**	M	M_C	P_W
SEQ	1	0.43±0.07	0.39±0.07	2.41±0.24	0.541	0.677	0.339
AYR	0	0.41±0.08	0.27±0.06	2.07±0.32	0.899	0.556	0.813
ROC	0	0.35±0.09	0.34±0.09	2.28±0.20	0.839	0.66	0.271
UNK	2	0.43±0.07	0.39±0.07	2.61±0.33	0.887	0.717	0.449
CEQ	3	0.43±0.08	0.40±0.08	3.00±0.45			
TSV	4	0.45±0.08	0.44±0.09	3.13±0.47			
FNQ	0	0.48±0.07	0.47±0.07	2.96±0.43			
CYP	2	0.50±0.08	0.48±0.08	3.24±0.48			

Estimates of the population genetic differentiation, Nei's unbiased genetic distance D is strongly correlated with allele richness (Pearson's correlation, R = 0.768, P = 0.026 < 0.05). In general, as populations diverge, the genetic diversity (measured as allele richness r) decreases. All captive populations are more genetically distinct from the wild populations; and there is a higher level of genetic diversity within wild populations than captive ones (Figure VI-1). AMOVA results showed that two groups (wild and captive) were significantly different (F_{CT} = 0.010, P = 0.025 < 0.05). The difference between populations within groups (F_{SC} = 0.061, P < 0.01) and within populations (F_{ST}

= 0.071, $P < 0.01$) was also significant. In all cases, most of the genetic variation (93%) was observed within populations (Table VI-2).

Table VI-2 Analysis of molecular variance (AMOVA) grouping populations of the Black-throated Finch according to sources (wild and captive)

Source of variance	df	Sum of squares	Variance components	Percentage of variation
Between sources (wild and captive)	1	33.135	0.034	1.04 (F_{CT} = 0.010, p = 0.025)
Between populations within sources	6	103.624	0.202	6.07 (F_{SC} = 0.061, p < 0.001)
Within populations	668	2061.259	3.086	92.89 (F_{ST} = 0.054, p < 0.001)
Total	675	2198.018	3.322	100

There was no evidence for a significant genetic bottleneck detected in any of the captive populations using the heterozygosity excess method. However, using *M*-ratio method, the SEQ population showed a significant signal of genetic bottleneck (*M*-ratio 0.541 < M_C = 0.677). When all captive birds were considered as one population, both methods failed to detect signals of significant genetic bottleneck (Table VI-1).

The individual based Bayesian clustering analysis in STRUCTURE identified two distinct genetic clusters (k = 2) taking into account all wild and captive populations (Figure VI-2). Notably, all wild populations belonged to one cluster and the captive population formed another, showing the differentiation between wild and captive birds. When considering the captive population alone, I identified 8 genetic clusters (k = 8). Birds that had known origins were roughly grouped into four clusters, mirroring their geographic origins. Birds that had unknown origins were approximately grouped into another four clusters with some individuals assigned to the same clusters of the birds that had known origins (Figure VI-2).

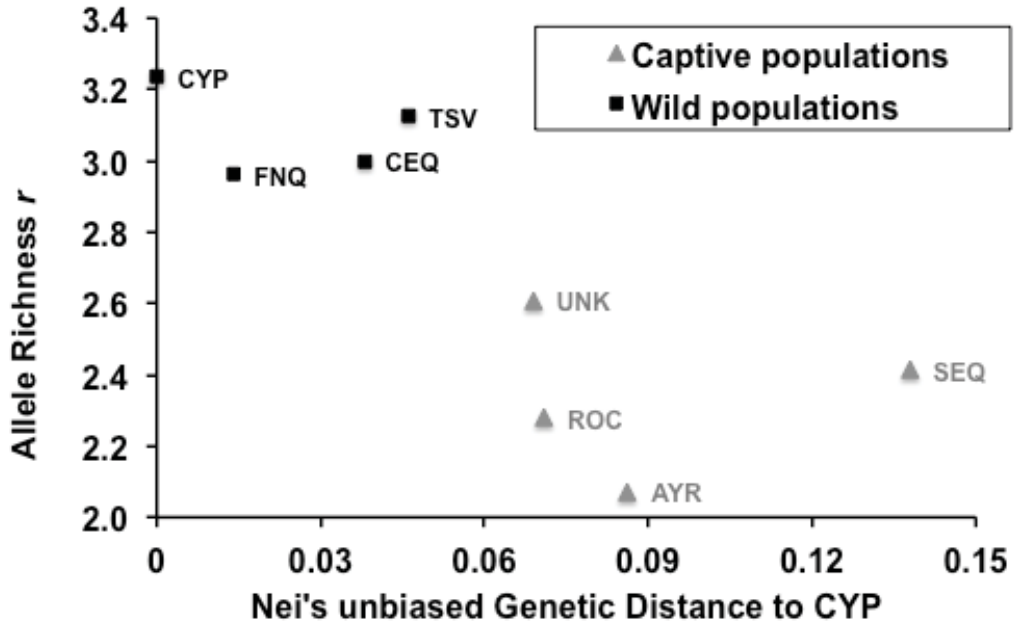

Figure VI-1 The genetic distance in relation to allele richness among captive (grey triangles) and wild (black squares) populations

Further population assignment tests showed that 75 out of 96 captive birds were self-assigned (78%). Out of the 21 individuals that were assigned to other populations, 13 were to CEQ population, 5 to the TSV population and 3 to FNQ. Only 7 out of the 49 captive birds of unknown origin were successfully assigned to wild populations with 3 to TSV population and 2 each to CEQ and FNQ populations (Table VI-2).

Effective population size (Ne), inbreeding and relatedness

Estimates of N_e varied widely depending on the methods used for each population. However, the N_e of the captive population was consistently smaller than the observed population size (N = 96): N_e = 10.1 using the linkage disequilibrium method; and N_e = 3.3 using the molecular coancestry method. The mutation-scaled effective population size showed a similar trend: it is the smallest in the AYR population (Θ = 0.001) and highest in the TSV population (Θ = 0.096). The averaged Θ is also significantly smaller

among captive populations (0.012) than that of the wild (0.071) (two-sample t test, $t_{(1),\,6} = 2.79$, $p = 0.016 < 0.05$) (Table VI-3).

Table VI-3 Comparison of estimates of effective population sizes (N_e) in each population using three different methods. Θ: mutation-scaled effective population size; *: captive populations

Region	Linkage disequilibrium		Molecular coancestry		Bayesian	
	N_e	95% CI	N_e	95% CI	Θ	95% CI
SEQ*	2.4	1.7-3.6	3.2	2.0-4.7	0.019	0.014-0.021
AYR*	1.0	0.8-1.5	1.7	1.2-2.4	0.001	0.000-0.002
ROC*	7.4	2.9-17.4	3.5	2.1-5.2	0.009	0.002-0.011
UNK*	11.4	7.4-17.0	8.3	3.1-16.2	0.019	0.002-0.021
CEQ	inf	173.6-inf	inf	inf-inf	0.088	0.075-0.095
TSV	75.3	52.5-115.2	9.6	2-23.1	0.096	0.093-0.100
FNQ	13.5	6.9-34.5	14.1	0.4-52	0.009	0.007-0.012
CYP	117.9	49-inf	28	4.6-71.8	0.091	0.085-0.097

The average inbreeding coefficient was significantly higher within the captive population ($F = 0.216$) compared with that in any of the wild populations (between 0.099 and 0.130). The population pairwise comparisons showed no significant differences in the level of inbreeding between all wild populations. All statistical significance was determined by 95% and 99% confidence intervals (Table VI-4).

The averaged relatedness estimate was slightly higher among captive birds ($R_r = 0.176$) than wild populations ($R_r = 0.145$). The individual relatedness within population is greatest in FNQ with an average of 0.184, followed by the captive population ($R_r = 0.176$). However, they were not significantly different (estimate of the difference fell within 95% confidence intervals). Similarly, the level of individual relatedness was not significantly different between CYP and CEQ populations. The individual relatedness between populations showed that FNQ and CYP are more related that any other populations ($R_r = 0.131$ and all other R_r values were less than 0.1). In both the captive and FNQ populations, 40-50% of the dyads (pairs of individuals) are distantly related

(R_r < 0.1) and about 8% are closely related; whereas in other wild populations, more than 50% of the dyads were distantly related and less than 2% were closely related (Figure VI-3).

Table VI-4 Pairwise comparisons of the inbreeding coefficient (*F*) between and within each Black-throated Finch population. Numbers along the diagonal are *F* values within each population and variances are summarised in brackets. Numbers below the diagonal are *F* values between each population pair. * indicates significant difference with 95% confidence interval and ** indicates significant difference with 99% confidence interval

	TSV	**CEQ**	**FNQ**	**CYP**	**CAP**
TSV	0.099 (0.014)				
CEQ	0.031	0.130 (0.020)			
FNQ	0.022	-0.009	0.121 (0.019)		
CYP	0.009	0.022	0.013	0.108 (0.012)	
CAP	0.117 **	0.085 **	0.095*	0.108 **	0.216 (0.043)

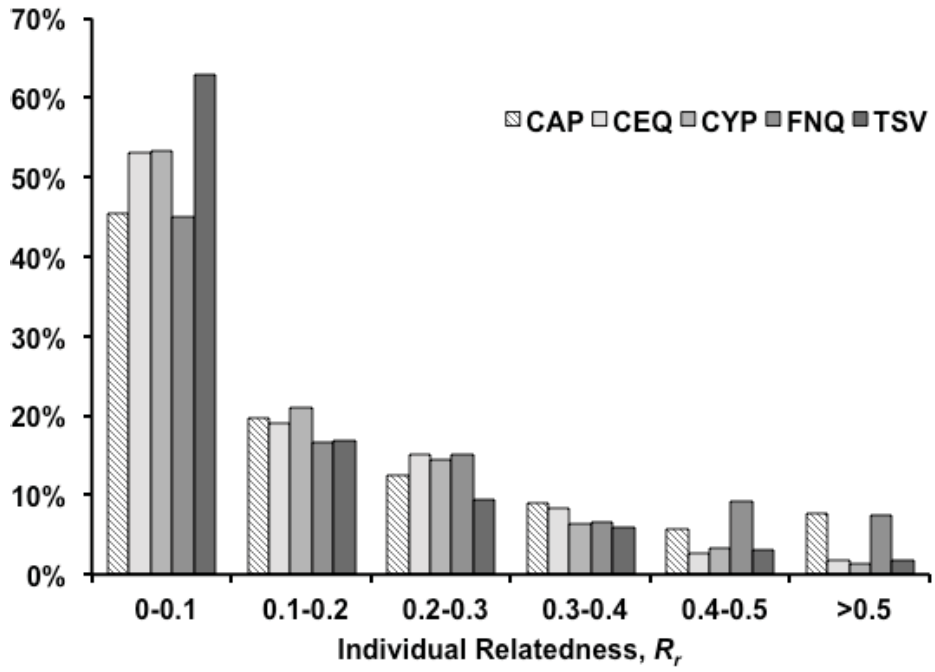

Figure VI-2 Comparison of the individual relatedness among wild (CEQ, CYP, FNQ and TSV) and captive (CAP) populations. Each bar represents the percentage of dyads in the relevant population

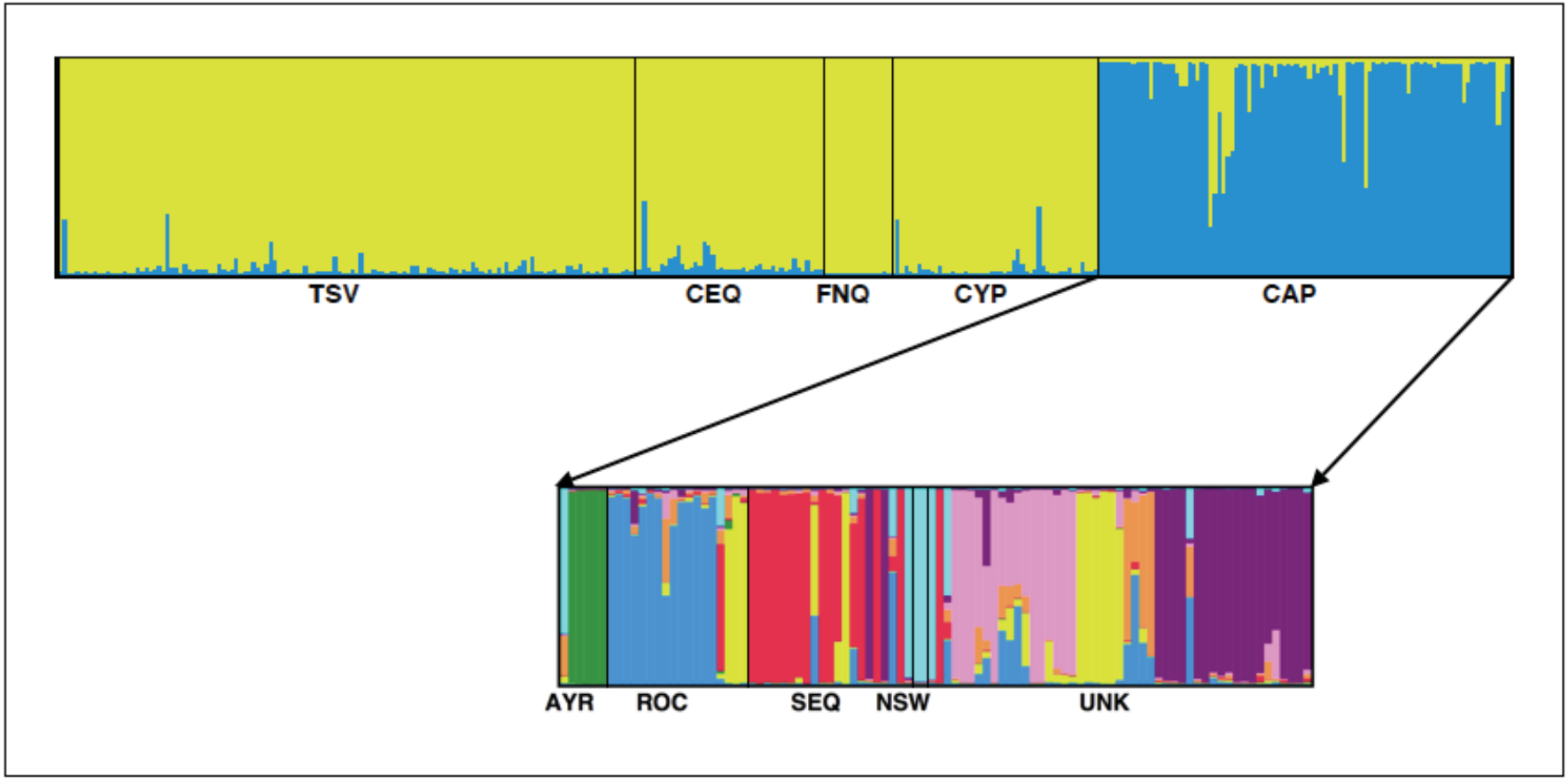

Figure VI-3 Individual membership coefficients derived from Bayesian inference of genetic structure across all captive and wild Black-throated Finch populations using admixture model (top, $k = 2$); and among all captive populations using admixture model (bottom, $k = 8$). A single vertical line represents an individual. Each colour represents a genetic cluster

DISCUSSION

It is essential to evaluate and compare the genetic diversity and viability of both the *in situ* and *ex situ* populations before any appropriate conservation strategies can be inferred. I examined conservation values of four major wild populations of the Black-throated Finch with 96 captive individuals using microsatellite markers. It is evident that the genetic diversity is moderately higher in wild populations than it is among sampled captive birds from various sources. Wild and captive populations of the finch are also genetically differentiated. Wild birds showed lower levels of inbreeding and individual relatedness than the captive population. This work provides genetic information useful for developing *ex situ* conservation strategies of the species.

Significant differentiation between captive and wild populations

Using variable microsatellite markers, I was able to detect significant differences between captive and wild populations of the Black-throated Finch. The genetic diversity measured as allele richness and observed heterozygosity, was significantly higher in wild populations than in captive ones. This is probably due to genetic drift in captivity and/or the larger effective population size in the wild. Typically, genetic drift occurs in small populations, where rare alleles are more likely to be lost. The result of this process reduced genetic diversity and increased genetic differentiation (Herdrick 2011). Wild populations of the Black-throated Finch showed modest levels of genetic differentiation across its current distribution (Chapter IV) and the estimates of N_e were significantly larger than captive populations demonstrated by multiple methods. Therefore, it is possible that the high number of alleles per locus that exist in the wild cannot be maintained in captivity as a result of genetic drift, leading to the observed reduction in allele richness and heterozygosity. The significant genetic differentiation between the

wild and captive Black-throated Finch was also supported by the population pairwise comparisons and AMOVA (the overall higher values of Nei's unbiased genetic distance *D* among captive populations than wild ones and the significant F_{RT} value).

Genetic differentiation between captive and wild populations is common in many avian species. For example, the Zebra Finch, one of the most common aviary birds in the world, showed significant differentiation between wild and captive populations (F_{ST} = 0.062). There was also a reduction in the genetic diversity (captive r = 11.7 compared to wild r = 19.3) among captive populations across the world (Forstmeier et al. 2007).

The increased genetic differentiation is particularly apparent in threatened species, highlighting the importance of understanding these genetic characteristics when designing effective conservation strategies. For example, current captive stocks of the endangered White-headed Duck (*Oxyura leucocephala*) have suffered a significant loss of genetic diversity due to founder effects and/or genetic drift and were not suitable for reintroduction. As a result, the development of a more diverse captive population based on birds taken from different areas of the range has been strongly recommended (Munoz-Fuentes et al. 2008). Overall, the observed higher level of genetic diversity in wild Black-throated Finch populations indicates that the evolutionary potential of the wild birds is still greater than that of the captive ones, as expected.

Effective population sizes and inbreeding

For both direct estimates of the effective population size, in all captive populations of the Black-throated Finch and the combined captive populations, the N_e was less than 12. N_e, however, was significantly larger for wild populations than for those in any of the captive ones. Small N_e increases the risk of inbreeding. The classic "50/500"

conservation guideline states that a population with an $N_e < 50$ is vulnerable to the immediate effects of inbreeding depression with high risk of extinction; and a population with an $N_e < 500$ is vulnerable to extinction in the long term (Franklin 1980). However, it is widely believed that populations must be higher than implied by the "50/500 rule" in order to survive and maintain genetic diversity (Lande 1995, Reed and Bryant 2000). It is therefore evident that the effective population sizes of the captive Black-throated Finch are too small to maintain high levels of genetic diversity. This is also supported by the fact that the level of inbreeding observed among captive birds ($F = 0.216$) is almost double that among wild birds (F ranged between 0.099 and 0.130).

Although the actual cost of inbreeding in populations of animals remains largely unknown, it is suggested that when the level of inbreeding is above intermediate levels ($F = 0.3 – 0.4$), there is an increased probability of extinction (Frankham 1995). The inbreeding coefficients of both wild and captive populations have not reached this threshold, indicating that the viability of all populations of the Black-throated Finch is unlikely to have been significantly affected by inbreeding. However, it is evident that inbreeding depresses components of reproductive fitness in a population. This is particularly the case in captive animals. For example, inbreeding has depressed the fitness of virtually every species of livestock in fertility, birth weight, growth rate, disease resistance and productivity (Lacy 1993).

I did not measure the individual fitness of the Black-throated Finch to quantify the potential impact of increased inbreeding among captive populations due to limited resources and logistic constrains. However, observations of behavioural and morphological traits among captive birds made by various aviculturists suggest some level of fitness loss. For example, in captivity, the Black-throated Finch is considered to

be aggressive. Particularly, male birds were observed to be so restlessly fond of nest building that they frequently disturb females that are sitting on eggs, leading to females becoming egg-bound (Butler 1899, Forshaw et al. 2012). This aggression reduces the breeding success of captive birds, but it has not been observed in the wild. Morphologically, the Black-throated Finch has several recognised colour mutations (Kingston 2010). In Australia, birds with mutations represent about 15% of all Black-throated Finches listed in a national survey of native finches held in captivity in 2011 by the National Finch and Softbill Association (Fitt and Pace 2011). Such high rates of mutation are probably due to the increased level of inbreeding and/or selective hybridisation with other Australian native finch species, e.g. the Long-tailed Finch (*Poephila acuticauda*), the Masked Finch (*Poephila personata*), the Zebra Finch (*Taeniopygia guttata*) and the Double-barred Finch (*Taeniopygia bichenovii*) (Forshaw et al. 2012). Therefore, I argue that the increased level of inbreeding in conjunction with decreased fitness has reduced the genetic viability of the Black-throated Finch in captivity.

Individual assignments and relatedness

Individuals in the captive population are only slightly more related to one another than are members of wild populations. The pairwise relatedness analysis showed that more than two thirds of the dyads in each population had relatedness values less than 0.2, indicating that a majority of the individuals in both captivity and the wild are not closely related. This suggests that a captive breeding programme for reintroduction could source individuals from among captive populations. The advantage of using existing captive birds is that these individuals have already undergone some degree of domestication, which potentially reduces the stress level of birds and increases their

productivity. However, the current captive populations of the Black-throated Finch demonstrated reduced genetic diversity and small effective population sizes, increasing the chance of the loss of behaviour traits that are critical for adaptation to natural environments (Shepherdson 1994).

Furthermore, I found that only approximately 22% of individuals in captivity were successfully assigned to any of the major wild populations and over half of these individuals were assigned to the CEQ populations. The low level of assignment success to current wild populations may reflect the moderate level of colour mutations found among captive birds as discussed above. It also suggests that the origins of captive birds could be from locations other than the geographical regions I sampled for this study. According to the owners of the sampled captive birds, a large proportion of them were derived from individuals legally trapped in the early 1900s from Southern Queensland and northern New South Wales, a population now considered to be extinct in the wild. I assigned 32 of the 96 captive birds to SEQ, representing a third of the overall captive populations sampled. Other identified source populations for the captive birds include the Rockhampton region (ROC) in central Queensland and the Ayr region (AYR) in north Queensland, just south of Townsville. These wild populations were not strongly represented in sampled captive birds, but they are important in maintaining the genetic diversity of the Black-throated Finch in captivity.

Conservation values of captive birds and management implications

Ex situ conservation measures, e.g. captive breeding programmes, are considered to be complementary to *in situ* methods because they provide “insurance” against species extinction. Through reintroduction, they often play a crucial role in preventing critically endangered species from becoming extinct in the wild, given that functioning wild

populations can be established from the captive population. *Ex situ* strategies are usually the last resort to preserve the genetic diversity of species that are under drastic decline in the wild.

Wild populations of the Black-throated Finch have declined significantly in the past 50 years and the current conservation strategies have been largely *in situ*. Although, they are relatively common in captivity, my results showed that the genetic diversity was lower among the captive populations than the wild ones. A higher level of inbreeding was also detected among captive birds. The genetic differentiation between captive and wild birds were significant. Given the fact that the genetic diversity of the wild populations is still moderate and the genetic connectivity is still relatively high, *in situ* management strategies (such as maintaining habitat suitability and increasing habitat connectivity) should be considered a priority.

However, my study has identified potential values of captive birds for *ex situ* conservation management. First, I found evidence that birds originated from populations that are now extinct (southern Queensland population in particular) in the wild are still maintained in captivity. These particular birds are valuable for the genetic diversity of captive breeding stocks. Therefore, it is vital to ensure these individuals are kept and bred separately from other birds so that the distinct genetic lineages are not lost. Second, the level of relatedness among captive birds is similar to that found in the wild. Existing management strategies could be applied to manage individuals of the Black-throated Finch that are closely related or inbred in captivity. For example, the maximum avoidance of inbreeding (MAI) scheme where family sizes are equalised and females are mated to males of different subpopulations each year (Kimura and Crow 1963, Frankham et al. 2002); and the rotational breeding scheme where breeding circles

are established by providing males from each subpopulation for its neighbouring subpopulation (Windig and Kaal 2008). Third, I demonstrated that molecular data could be used to identify individual relatedness of the Black-throated Finch. In combination with the knowledge of birds with known origins, it is possible to establish detailed individual pedigree information using the genetic markers described in this study.

If a captive breeding programme for the purpose of wild reintroductions was going to be established in the future, it is important to work with captive breeders to retain genetic diversity of the declining Black-throated Finch. Specifically, I recommend the following steps: (1) establish a genetic structure of captive birds that are derivatives of wild populations of the Black-throated Finch including populations that are extinct in the wild; (2) define management units (populations that have different allele frequencies, but do not necessarily show fixed differences between them) based on more genetic data over a broader region and prioritise these units within the species; (3) assign captive individuals to defined management units following the methods used in this study; (4) select suitable individuals to establish a breeding stock for each unit; (5) collect additional wild individuals, if possible, to maximise genetic variability within each unit; and (6) apply management strategies mentioned above to minimise inbreeding and reassess genetic structure of the captive population regularly.

CHAPTER VII

General conclusion

(Photo credit: L. Stanley Tang)

Understanding the genetic processes of any declining species is vital to assess the viability of surviving populations. It is also an important step towards the implementation of the most appropriate conservation management strategies. Genetic studies of declining granivorous birds in Australia and specifically in the context of the Black-throated Finch (*Poephila cincta*) are rarely conducted. My project set out to examine the genetic viability of a threatened granivorous bird in Australia. Using the Black-throated Finch as a case study, my project examined key genetic processes involved in the decline of the bird as a result of habitat alteration in recent years. I also evaluated the conservation values of captive populations and made recommendations for the conservation of the Black-throated Finch. In my project, I sought to answer three key questions:

1. What is the level of genetic diversity and population structure of the Black-throated Finch across its current range?
2. Is there any association between landscape features and the spatial genetic structuring of the Black-throated Finch?
3. Are captive populations of the Black-throated Finch valuable for future re-introduction programmes?

I used both population and landscape genetic approaches with microsatellite and mitochondrial DNA markers to address these key questions at regional and fine spatial scales.

EMPIRICAL FINDINGS

Range-wide genetic structure and diversity

I sampled four populations spanning over 750km in northern Queensland, Australia. According to sighting records, these four populations represent the main of areas where the Black-throated Finch currently occurs. In particular, the Townsville region and central Queensland are the only known strong holds of the surviving southern subspecies in the wild. Based on 14 microsatellite markers, I found modest levels of genetic diversity (average allelic richness $r = 4.37 \pm 0.41$ standard error and average heterozygosity $H_O = 0.42 \pm 0.040$ standard error) with no bottleneck signature within these populations. The level of genetic diversity is similar across all populations at species level. However, populations of the northern subspecies were slightly more genetically diverse than those of the southern subspecies.

I also provided molecular evidence that the genetic structure of sampled populations of the Black-throated Finch are differentiated, particularly between northern and southern forms. The observed differentiation and sub-structuring of populations is likely due to the isolation caused by habitat fragmentation and as a result of restricted dispersal (see detailed discussion in Chapter IV).

Effects of landscape features

Radio tracking and banding data suggest that the Black-throated Finch does not move further than 30km (Rechetelo 2016). Changes in local environment may have a bigger impact on the survival of the species compared with more mobile species. The Townsville region has gone through intense land modifications for cattle grazing, agricultural and urban developments in the past 20 years. Sighting records of the Black-

throated Finch suggest that populations in the Townsville region have declined significantly and are fragmented as a result of habitat changes. Therefore, it is necessary to understand the association between the population genetic structure and the heterogeneous landscape that the bird lives in.

I sampled birds from sites that spanned no more than 20km between the two most distant sampling points. I found that local landscape features, including the presence of water bodies, vegetation structure and terrain are correlated with the genetic distance between individuals. I provided evidence that genetic structuring can be detected at fine scales for species that have limited dispersal ability. Although weak, I also demonstrated the effects of open waters on the spatial genetic structure of the Black-throated Finch in northeastern Queensland. In this case, the Ross River Dam has a surface area of 82km^2; it is likely to have acted, to some extent, as a barrier of gene flow separating birds into two genetic groups. The Ross River Dam was built only about 40 years ago for the purpose of flood control and water supply to the Townsville region and the presence of genetic differentiation was not overwhelmingly significant. Nonetheless, it demonstrated that the genetic structure of the Black-throated Finch could be influenced by local landscape features and highlighted the potential for open water bodies to act as barriers to the gene flow.

Conservation values of *ex situ* populations

I evaluated the genetic diversity and structuring of captive individuals of the Black-throated Finch sourced from various breeding stocks held by aviculturists in Australia. The captive populations showed lowered levels of genetic diversity compared with wild populations. Although no evidence of high levels of inbreeding was detected, I found that the effective population sizes were smaller and individuals were more related to

each other in captive populations than they were in the wild. The genetic differentiation was also significant between captive and wild populations.

Due to the significant differentiation between captive and wild populations of the Black-throated Finch, I recommended that conservation efforts should focus mainly on *in situ* populations at present. However, based on the fact that the inbreeding depression was not detected in captivity and the genetic distinctiveness of many captive individuals, it is valuable to use captive individuals and to work with breeders to retain genetic diversity of the captive population (see Chapter VII for detailed discussion).

IMPLICATIONS FOR CONSERVATION MANAGEMENT

Using multiple genetic approaches, my thesis has some critical implications for the conservation management of the Black-throated Finch. First, I recommend that *in situ* conservation strategies should be given priority. This is based on the fact that current major populations of the Black-throated Finch have not yet experienced severe genetic bottlenecks and the level of genetic diversity is similarly moderate across all populations. Specifically, the conservation effort should focus on the following areas.

a) *Continuous monitoring of population trends to allow quick detection of significant decline.* Currently, there are only limited regular surveys conducted each year to monitor the Black-throated Finch. The on-going annual waterhole counts has been the major source of population estimates in the Townsville region (Black-throated Finch Recovery Team 2007). Other intensive field surveys and monitoring work have been conducted by environmental consulting companies for various development projects in both Central and North Queensland (Natural Resource Assessment Environmental Consultants 2005, 2007, GHD Australia 2010, 2012).

Information collected from these surveys is nonetheless crucial, but a more intensive, regular and systematic survey programme should be used to monitor population trends in multiple locations, particularly the Townsville region and Central Queensland – the two known remaining strong-holds of the southern subspecies.

b) *Increasing habitat suitability and connectivity* My landscape genetic analysis indicated that large open water bodies and areas with dense understorey may reduce gene flow of the Black-throated Finch. Maintaining areas with critical water sources, particularly in the dry season, and controlling invasive grass species to create mosaic ground cover with open patches are essential to increase the habitat suitability and connectivity. It is also important to combine my findings with other ecological studies (e.g. movements and breeding ecology) to determine more comprehensive habitat requirements for the Black-throated Finch, so that appropriate measures can take place for the future land management.

c) *Applying landscape genetics analysis.* I used the Townsville population to investigate the association between local landscape features and population genetic structuring. The landscape genetics technique I used may be applied to other areas that are of conservation concern. For example, many Black-throated Finch habitats are threatened by land development, particularly for coal mines (GHD Australia 2012). Landscape genetics analysis could be used to identify potential corridors to gene flow of the bird. It could also reveal local landscape features that are associated with genetic structuring. This spatial genetic information could aid in relevant environmental impact assessments and the conservation of local Black-throated Finch populations.

d) *Ex situ conservation strategies.* My results demonstrated that captive individuals of the Black-throated Finch have potential for the establishment of captive breeding programmes. The genetic diversity was only slightly lower than that of wild populations. Many individuals in captivity represented lineages originated from now extinct populations in the wild. *Ex situ* conservation efforts should be focused on maintaining the genetic diversity of captive populations; ensuring individuals originated from unique lineages are kept and bred separately; and minimising inbreeding. A detailed molecular and genotypic analysis could also be carried out to build a comprehensive pedigree network, so that viable and healthy individuals can be selected for the maintenance of breeding stocks.

RECOMMENDATIONS FOR FUTURE RESEARCH

Due to time and funding constraints, as well as the rarity of the Black-throated Finch and the difficulty in sampling, I was able to collect only a limited number of samples. Despite providing some crucial evidence and information for the conservation of the bird, I acknowledge that there are still many areas that require further research in order to produce a more comprehensive and long-term conservation strategy for the survival of the Black-throated Finch.

First, I recommend that more populations should be sampled. It is very time-consuming to discover and establish a reliable site where birds visit frequently and are easy to trap at the same time. In the future, it is important to survey more potential waterholes and other possible sites for sample collections. Particularly, the priority should be given to establish more sampling sites in Central Queensland, where the largest numbers of the Southern Black-throated Finch are present. In the northern range of the species, more

sites representing different geographical locations across the Cape York Peninsula to Mareeba Wetlands in Far North Queensland would be ideal. In the Townsville region, for fine-scale genetic analysis, more sampling locations could be established to the north and southwest of the Ross River Dam, as well as a few sites further away from the dam, e.g. north of the Townsville city. With more sampling locations included, it would be easier to construct a more detailed genetic structure and to estimate more accurately the genetic diversity of the surviving populations of the Black-throated Finch.

Second, I recommend that more genetic markers should be used to increase the statistical power so that more detailed genetic structuring can be detected. Specifically, it would be helpful to use large numbers of single nucleotide polymorphisms (SNPs) in combination with microsatellite and mitochondrial markers to map out an allele spectrum across the entire genome for each population. This would provide a higher resolution for detecting subtle population genetic structuring of the Black-throated Finch. Using universal DNA markers on sex chromosomes can also be important to directly examine sex-linked characteristics, e.g. female-biased dispersal and parental analysis.

Third, I recommend sourcing more historical samples from museum collections to examine temporal changes to determine if the moderate level of genetic diversity was historically present or due to recent habitat fragmentation. Knowing both the temporal and spatial patterns of the genetic structure would provide a much more comprehensive understanding of the changes in genetic diversity of the Black-throated Finch. Samples collected in captivity could include more individuals from different sources. For example, birds from wildlife parks and zoos in Australia and overseas; birds with mutations and different colour morphs; as well as possible interspecific hybrids. It is

possible to establish a complete genetic database of the Black-throated Finch in captivity so that healthy and viable breeding stocks can be maintained and sourced if captive breeding programmes were required.

Last, I suggest that demographic data, including survival rates of each life stage, individual fitness, life cycles, clutch sizes, and growth rates, of the Black-throated Finch should be collected in detail. In combination with the genetic information I have provided in this thesis, it would then be possible then to perform an robust population viability analysis, so that the extinction risk could be estimated and the overall future population performance could be projected.

Overall, this project examined the genetic diversity, population structuring and the landscape genetics of the Black-throated Finch. Results revealed valuable information on the current stage of the species both in the wild and in captivity. Based on this information, I have provided recommendations to the conservation of the Black-throated Finch in the future.

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APPENDICES

APPENDIX A

Field Protocol: Avian blood collection using FTA® cards

1. Unfold one wing and hold tightly to avoid the bird moving.
2. Expose the venepuncture site by using alcohol (70%) to wet the feathers, then separate the feathers with fingers; the ulnar vein should be seen as it passes over the ventral side of the elbow.
3. Pluck some feathers if necessary to improve visibility; plucked feathers should be placed in ziplock bags with clear labels.
4. Apply light pressure on the vein towards the heart to temporarily block blood flow so that the vein can be easily located.
5. Carefully use the hypodermic needle (25 or 26 gauge) to prick the vein so that the blood emerges onto the skin.
6. Use heparinised capillary tube (~70μL in capacity) to carefully absorb blood.
7. Collect 1/3 – 1/2 tube of blood for each bird and transfer the blood to the Classic FTA® card (Whatman, USA). Blood spots should be dried and absorbed by the card in shaded areas and placed in paper envelopes for safe and easy storage.
8. Apply pressure to the vein using cotton wool for 30-60 seconds to prevent excessive bleeding and hematoma formation.
9. Check if the bird is in good condition and if so, release on site; otherwise put the bird back into the bag for 30 minutes to allow resting before release.

APPENDIX B

Laboratory Protocol: DNA extraction from avian blood stored on FTA© cards using ISOLATE Genomic DNA Kit (BIOLINE, Australia)

This protocol is modified from the product manual of ISOLATE DNA kits of BIOLINE. It is designed for isolating genomic DNA from FTA cards/filter papers.

Equipment

- Micro punch, 1.2mm (can be replaced by normal hole punch or fine scissors)
- Smooth fine-point tweezers
- Three beakers filled with diluted bleach, ethanol, and water respectively
- Laboratory-standard tissue paper
- Shaking water bath or oven
- 1.5mL tubes (one per sample)
- Microcentrifuge with rotor for 1.5mL and 2mL tubes
- Two waste collection plates
- Full set of pipettes and autoclaved tips

Solutions

<!> indicates a hazardous substance, further details give below

- Ethanol, 96-100% **<!> flammable<!>**
- ddH_2O
- ISOLATE Genomic DNA kit (components as tabled below)

Regent	**10 preps**	**50 preps**	**250 preps**
Lysis Buffer D **<!> flammable, irritant<!>**	5mL	25mL	120mL
Binding Buffer D **<!> flammable, irritant<!>**	2 × 2mL	15mL	70mL
Proteinase K	0.3mL	1.5mL	5 × 1.5mL

Wash Buffer D	6mL	24mL	2 × 60mL
Elution Buffer	2 × 2mL	25mL	110mL

Hazards & Safety considerations

Hazard (%)	Risk Phrases	Safety Phrases/Precautions
Lysis Buffer D <!>flammable<!> <!>irritant<!> R11, R36, R67 S7, S16, S24/25, S26	Highly flammable, Irritating to eyes, Vapours may cause drowsiness and dizziness	Keep container tightly closed; Keep away from sources of ignition; Avoid contact with skin and eyes; In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
Binding Buffer D <!>flammable<!> <!>irritant<!> R11, R36, R67 S7, S16, S24/25, S26	Highly flammable, Irritating to eyes, Vapours may cause drowsiness and dizziness	Keep container tightly closed; Keep away from sources of ignition; Avoid contact with skin and eyes; In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
Proteinase K R36/37/38, R42 S22, S24, S26, S36/37	Irritating to eyes, respiratory system and skin; May cause sensitization by inhalation	Do not breathe dust; Avoid contact with skin; In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; Wear suitable protective clothing and gloves
Ethanol, 96% <!>flammable<!> R11 S2, S7, S16	Highly flammable	Keep out of the reach of children; Keep container tightly closed; Keep away from sources of ignition

Minimum personal protective equipment (PPE)

- Long sleave laboratory coat & latex gloves throughout.

Hazardous Waste Disposal

- Dispose of tubes and tips via MEEL approved (ChemWatch Labelled) containers.
- Dispose of all wet waste via dilution with tap water to <20% and dispose of via fume hood sink flushing for at least 2 minutes (<50mL) or 20 minutes (> 50mL).

Important considerations

- Use sterile equipment only. Be very aware of any possible sources of contamination- particularly genetic contamination between samples.
- Use a control extraction.
- Carefully label all tubes throughout the extraction.
- Add 96-100% ethanol **<!>flammable<!>** to the Wash Buffers as indicated on the bottles, mix thoroughly.
- Reconstitute lyophilised Proteinase K in water, 0.3mL for each 10 preps. Aliquot to avoid freeze thawing.
- Prepare a 50°C shaking water bath or heating block for Proteinase K lysis cell membranes.

Method

1. Punch up to 5 small pieces (1.2mm diametre) from the FTA card, place in 1.5mL tube.
2. Add 200µL Lysis Buffer D and 15µL Proteinase K. Mix by vortexing.
3. Incubate at 50ºC overnight and vortex intermittently to disperse the sample.
4. Centrifuge at 10,000 × g (12,000rpm) for 1 minute. Transfer the supernatant to another 1.5mL tube.
5. Add 200µL Binding Buffer D to the sample. Mix well by vortexing for 15 seconds.
6. Transfer the sample to Spin Column D placed in a 2mL Collection Tube.
7. Centrifuge at 10,000 × g (12,000rpm) for 2 minutes. Discard the Collection Tube and place Spin Column D in a new Collection Tube.
   (Ensure there is no lysate remaining on Spin Column D. If required, centrifuge Spin Column again until all liquid has passed through the membrane.)
8. Add 350µL Wash Buffer D.

9. Centrifuge at 10,000 × g (12,000rpm) for 1 minute. Discard the filtrate and reuse the Collection Tube.
10. Repeat steps 8 and 9.
11. Centrifuge at maximum speed for 2 minutes to remove all traces of ethanol. Discard the Collection Tube.
12. Place Spin Column D into a 1.5mL Elution Tube.
13. Add 50µL Elution Buffer directly to the Spin Column membrane and incubate at room temperature for 1 minute.
14. Centrifuge at 6,000 × g (8,000rpm) for 1 minute to elute DNA.
    (Use a lower volume of Elution Buffer if a high concentration of DNA is required. Alternatively, perform two elution steps with an equal volume of Elution Buffer to increase the yield)
    (Repeat step 13 – 14 four times until the final Elution Buffer is 200µL)
15. The isolated DNA is ready for use in downstream applications or for storage at -20°C. For long-term storage, freeze at -70°C.
16. Aliquot DNA into three tubes of 50µL × 2 (working stocks, -20°C) and 100µL (long term storage, -80°C).

APPENDIX C

Laboratory Protocol: DNA extraction from avian blood cells using ISOLATE II Blood DNA Kit (BIOLINE, Australia)

This protocol is modified from the product manual of ISOLATE II Blood DNA kits of BIOLINE. It is designed for isolating genomic DNA from avian blood cells.

Equipment

- Shaking water bath or oven
- 1.5mL tubes (one per sample)
- Microcentrifuge with rotor for 1.5mL and 2mL tubes
- Two waste collection plates
- Full set of pipettes and autoclaved tips

Solutions

<!> indicates a hazardous substance, further details give below

- Ethanol, 96-100% **<!>flammable<!>**
- ddH_2O
- ISOLATE II Blood DNA kit (components as tabled below)

Regent	**10 preps**	**50 preps**	**250 preps**
Buffer G1 **<!>irritant<!>**	3.2mL	10mL	50mL
Buffer G2	0.8mL	2.5mL	12.5mL
Proteinase K Buffer PR	0.8mL	1.8mL	8mL
Proteinase K (lyophilised)	6 mg	30 mg	2 × 75 mg
Wash Buffer GW1 **<!>flammable, irritant<!>**	6mL	30mL	2 × 75mL
Wash Buffer GW2	4mL	7mL	2 × 20mL
Elution Buffer G	4mL	13mL	60mL

Hazards & Safety considerations

Hazard (%)	Risk Phrases	Safety Phrases/Precautions
Buffer G1 <!>irritant<!> R11, R36, R67 S7, S16, S24/25, S26	Irritating to eyes, Vapours may cause drowsiness and dizziness	Keep container tightly closed; Avoid contact with skin and eyes; In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
Wash Buffer GW1 <!>flammable<!> <!>irritant<!> R11, R36, R67 S7, S16, S24/25, S26	Highly flammable, Irritating to eyes, Vapours may cause drowsiness and dizziness	Keep container tightly closed; Keep away from sources of ignition; Avoid contact with skin and eyes; In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
Proteinase K R36/37/38, R42 S22, S24, S26, S36/37	Irritating to eyes, respiratory system and skin; May cause sensitization by inhalation	Do not breathe dust; Avoid contact with skin; In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; Wear suitable protective clothing and gloves
Ethanol, 96% <!>flammable<!> R11 S2, S7, S16	Highly flammable	Keep out of the reach of children; Keep container tightly closed; Keep away from sources of ignition

Minimum personal protective equipment (PPE)

- Long sleave laboratory coat & latex gloves throughout.

Hazardous Waste Disposal

- Dispose of tubes and tips via MEEL approved (ChemWatch Labelled) containers.
- Dispose of all wet waste via dilution with tap water to <20% and dispose of via fume hood sink flushing for at least 2 minutes (<50mL) or 20 minutes (> 50mL).

Important considerations

- Use sterile equipment only. Be very aware of any possible sources of contamination- particularly genetic contamination between samples.
- Use a control extraction.
- Carefully label all tubes throughout the extraction.
- Blood samples should be stored at -20ºC or -70ºC for long-term storage; room temperature or 4ºC storage is only for short-term usage for up to several days.
- Transfer all contents of Buffer G1 to Buffer G2, mix well and label Buffer G3
- Add 80mL × 2 ethanol (96-100%) to Wash Buffer GW2 concentrate and label "ethanol added"
- Add 3.35mL × 2 Proteinase K Buffer PR to the lyophilised Proteinase K (brief centrifuge first before adding buffer)
- Proteinase K solution is stable at -20ºC for 6 months – make one solution at a time

Method

Protocol volumes are for 16 samples

1. Set incubator to 70ºC and preheat Elution Buffer G to 70ºC.
2. Prepare and label 16 × 1.5mL microcentrifuge tubes.
3. Mix 400µl (25µl × 16) Proteinase K solution with 3200µl (200µl × 16) Lysis Buffer G3
4. Add 225µl mixed solution to each sample.
5. Incubate samples at 70ºC for 1h and vortex vigorously 4 times.
   (The lysate should turn brownish during incubation.)
6. Add 210µl ethanol (96-100%) to each sample and vortex.
7. Transfer the sample (all lysate) to a spin column in a collection tube and centrifuge at 11,000g for 2 minutes. Place column in a new collection tube (2mL).
8. Add 500µl Wash Buffer GW1 and centrifuge at 11,000 × g for 1 minute. Place column in a new collection tube (2mL).
9. Add 600µl Wash Buffer GW2 and centrifuge at 11,000 × g for 2 minutes. Discard flow-through and re-use the collection tube.
10. Centrifuge at 11,000 × g for a further 1 minute to remove all ethanol residuals. Discard flow-through and put the column in a new 1.5mL microcentrifuge tube.

11. Add 50µl preheated Elution Butter G (70ºC) directly onto the silica membrane and incubate at room temperature for 5 minutes.
12. Centrifuge 11,000 × g for 1 minute.
    (Repeat step 10-11 to allow high yield & high concentration of DNA.)

APPENDIX D

Laboratory Protocol: PCR product clean-up using MicroCLEAN Kit (MICROZONE, UK)

Equipment

- Centrifuge or microfuge
- Pipettes and tips
- PCR plates or strip tubes

Solutions

<!> indicates a hazardous substance, further details give below

- MicroCLEAN solution <!> **irritant**<!>

Hazards & Safety considerations

Hazard (%)	Risk Phrases	Safety Phrases/Precautions
MicroCLEAN (Microzone Limited) <!> **irritant**<!>	Harmful if swallowed, Irritant, Irritating to eyes, respiratory system and skin	In case of contact with eyes, rinse immediately with plenty of water for at least 15 min. In case of contact with skin, immediately wash skin with soap and copious amounts of water If inhaled, move to fresh air and monitor breathing. Administer first aid or seek medical advice if breathing is absent or difficult. If swallowed, wash out mouth with water if person is conscious, and seek medical aid.

Minimum personal protective equipment (PPE)

- Long sleeve laboratory coat & latex gloves throughout.

Hazardous Waste Disposal

None

Important considerations

- Use sterile equipment only
- Store at 4°C

Method

1. Add equal volume of MicroCLEAN <!> **irritant**<!> to PRC product.
2. Mix by pipetting at least 10-15 times.
3. Leave at room temperature for 5 minutes.

If using tubes:

1. Spin at high speed (10,000 – 13,000 × g) in microfuge for 7 minutes.
2. Remove supernatant.
3. Spin briefly again and remove dregs.
4. Resuspend pellet in the appropriate amount of dH_2O.
5. Leave for 5 minutes to rehydrate DNA.

If using plates:

1. Spin at 3200 × g (4000 rpm in Beckman centrifuge with swing bucket rotor) for 40 minutes.
2. Place plate upside down onto tissue paper in the centrifuge holder.
3. Pulse centrifuge to 200 × g for 2 minutes.
4. Resuspend pellet in the appropriate volume of dH_2O.
5. Leave for 5 minutes to rehydrate DNA and vortex to mix.

APPENDIX E

Raw Data I: details of the Black-throated Finch samples acquired from the wild used in the analysis

Region codes:

TSV: Townsville Coastal Plains, Queensland

CEQ: Central Queensland

FNQ: Far North Queensland

CYP: Cape York Peninsula, Queensland

Site codes:

LAP: Ladham Park, Ross River Dam west

CLW: Clear Water, Ross River Dam west

DOD: Dottrel Dam, Ross River Dam south

FOD: Ford's Dam, Ross River Dam southeast

MAD: Mango Dam, Ross River Dam southeast

ANC: Antill Creek, Ross River Dam east

ARC: Sunbird Creek, Ross River Dam north

SAMPLE ID	RING CODE[‡]	REGION	SITE	SUBSPECIES
kBTFI001	02647271	TSV	LAP	*cincta*
kBTFI002	02647272	TSV	LAP	*cincta*
kBTFI003	02647273	TSV	LAP	*cincta*
kBTFI004	02647274	TSV	LAP	*cincta*
kBTFI005	02647275	TSV	LAP	*cincta*
kBTFI006	02647276	TSV	LAP	*cincta*
kBTFI007	02647277	TSV	LAP	*cincta*

[‡] Leg ring coding system followed the standards set out by the Australian Bird and Bat Banding Scheme (ABBBS).

SAMPLE ID	RING CODE‡	REGION	SITE	SUBSPECIES
kBTFI008	02647278	TSV	LAP	*cincta*
kBTFI009	02647279	TSV	LAP	*cincta*
kBTFI010	02647280	TSV	LAP	*cincta*
kBTFI011	02647281	TSV	LAP	*cincta*
kBTFI012	02647282	TSV	LAP	*cincta*
kBTFI013	02647283	TSV	LAP	*cincta*
kBTFI014	02647284	TSV	LAP	*cincta*
kBTFI015	02647285	TSV	LAP	*cincta*
kBTFI016	02647286	TSV	LAP	*cincta*
kBTFI017	02647287	TSV	LAP	*cincta*
kBTFI018	02647288	TSV	LAP	*cincta*
kBTFI019	02647289	TSV	LAP	*cincta*
kBTFI020	02647290	TSV	LAP	*cincta*
kBTFI021	02647291	TSV	LAP	*cincta*
kBTFI022	02647292	TSV	LAP	*cincta*
kBTFI023	02647293	TSV	LAP	*cincta*
kBTFI024	02647294	TSV	LAP	*cincta*
kBTFI025	02647311	FNQ	LFN	*atropygialis*
kBTFI026	02647312	FNQ	LFN	*atropygialis*
kBTFI027	02647316	FNQ	LFN	*atropygialis*
kBTFI028	02647317	FNQ	LFN	*atropygialis*
kBTFI029	02647320	FNQ	LFN	*atropygialis*
kBTFI030	02647321	FNQ	LFN	*atropygialis*
kBTFI031	02647322	FNQ	LFN	*atropygialis*
kBTFI032	02647323	FNQ	LFN	*atropygialis*
kBTFI033	02647324	FNQ	LFN	*atropygialis*
kBTFI034	02647325	FNQ	LFN	*atropygialis*
kBTFI035	02647326	FNQ	LFN	*atropygialis*
kBTFI036	02647327	FNQ	LFN	*atropygialis*
kBTFI037	02647328	FNQ	LFN	*atropygialis*
kBTFI038	02647329	FNQ	LFN	*atropygialis*
kBTFI039	02647330	FNQ	LFN	*atropygialis*
kBTFI040	02647331	FNQ	LFN	*atropygialis*
kBTFI041	02647332	FNQ	LFN	*atropygialis*
kBTFI042	02647333	FNQ	LFN	*atropygialis*
kBTFI043	02647334	FNQ	LFN	*atropygialis*
kBTFI044	02647335	FNQ	LFN	*atropygialis*
kBTFI045	02647336	FNQ	LFN	*atropygialis*
kBTFI046	02647337	FNQ	LFN	*atropygialis*
kBTFI047	02647338	FNQ	LFN	*atropygialis*
kBTFI048	02647339	FNQ	LFN	*atropygialis*
kBTFI049	02647870	FNQ	LFN	*atropygialis*
kBTFI050	02647871	FNQ	LFN	*atropygialis*
kBTFI051	02647872	FNQ	LFN	*atropygialis*
kBTFI052	02647873	FNQ	LFN	*atropygialis*
kBTFI053	02647874	FNQ	LFN	*atropygialis*
kBTFI054	02647875	FNQ	LFN	*atropygialis*

SAMPLE ID	RING CODE‡	REGION	SITE	SUBSPECIES
kBTFI055	02647876	FNQ	LFN	*atropygialis*
kBTFI056	02647877	FNQ	LFN	*atropygialis*
kBTFI057	02647878	FNQ	LFN	*atropygialis*
kBTFI058	02647879	FNQ	LFN	*atropygialis*
kBTFI059	02647880	FNQ	LFN	*atropygialis*
kBTFI060	02647881	FNQ	LFN	*atropygialis*
kBTFI061	02647882	FNQ	LFN	*atropygialis*
kBTFI062	02647886	FNQ	LFN	*atropygialis*
kBTFI063	02647887	FNQ	LFN	*atropygialis*
kBTFI064	02647888	FNQ	LFN	*atropygialis*
kBTFI065	02647890	FNQ	LFN	*atropygialis*
kBTFI066	02647891	FNQ	LFN	*atropygialis*
kBTFI067	02647892	FNQ	LFN	*atropygialis*
kBTFI068	02647893	FNQ	LFN	*atropygialis*
kBTFI069	02647895	FNQ	LFN	*atropygialis*
kBTFI070	02647896	FNQ	LFN	*atropygialis*
kBTFI071	02647897	FNQ	LFN	*atropygialis*
kBTFI072	02647898	FNQ	LFN	*atropygialis*
kBTFI073	02647947	TSV	LAP	*cincta*
kBTFI074	02647948	TSV	LAP	*cincta*
kBTFI075	02647949	TSV	LAP	*cincta*
kBTFI076	02647950	TSV	LAP	*cincta*
kBTFI077	02647951	TSV	LAP	*cincta*
kBTFI078	02647952	TSV	LAP	*cincta*
kBTFI079	02647953	TSV	LAP	*cincta*
kBTFI080	02647954	TSV	LAP	*cincta*
kBTFI081	02647955	TSV	LAP	*cincta*
kBTFI082	02647956	TSV	LAP	*cincta*
kBTFI083	02647957	TSV	LAP	*cincta*
kBTFI084	02647958	TSV	LAP	*cincta*
kBTFI085	02647959	TSV	LAP	*cincta*
kBTFI086	02647960	TSV	LAP	*cincta*
kBTFI087	02647961	TSV	LAP	*cincta*
kBTFI088	02647962	TSV	LAP	*cincta*
kBTFI089	02647963	TSV	LAP	*cincta*
kBTFI090	02647964	TSV	LAP	*cincta*
kBTFI091	02647965	TSV	LAP	*cincta*
kBTFI092	02647966	TSV	LAP	*cincta*
kBTFI093	02647967	TSV	LAP	*cincta*
kBTFI094	02647968	TSV	LAP	*cincta*
kBTFI095	02647969	TSV	LAP	*cincta*
kBTFI096	02647970	TSV	LAP	*cincta*
wBTFI001	02692503	TSV	CLW	*cincta*
wBTFI002	02692534	TSV	CLW	*cincta*
wBTFI003	02692567	TSV	DOD	*cincta*
wBTFI004	02692568	TSV	DOD	*cincta*
wBTFI005	02692569	TSV	DOD	*cincta*

SAMPLE ID	RING CODE‡	REGION	SITE	SUBSPECIES
wBTFI006	02692570	TSV	DOD	*cincta*
wBTFI007	02692571	TSV	DOD	*cincta*
wBTFI008	02692572	TSV	DOD	*cincta*
wBTFI009	02692573	TSV	CLW	*cincta*
wBTFI010	02692574	TSV	CLW	*cincta*
wBTFI011	02692575	TSV	CLW	*cincta*
wBTFI012	02692576	TSV	CLW	*cincta*
wBTFI013	02692577	TSV	CLW	*cincta*
wBTFI014§	Not banded	TSV	CLW	*cincta*
wBTFI015	02692590	TSV	ANC	*cincta*
wBTFI016	02692591	TSV	ANC	*cincta*
wBTFI017	02692592	TSV	ANC	*cincta*
wBTFI018	02692594	TSV	ANC	*cincta*
wBTFI019	02692595	TSV	ANC	*cincta*
wBTFI020	02692670	TSV	MAD	*cincta*
wBTFI021	02692671	TSV	MAD	*cincta*
wBTFI022	02692672	TSV	MAD	*cincta*
wBTFI023	02692673	TSV	MAD	*cincta*
wBTFI024	02692674	TSV	FOD	*cincta*
wBTFI025	02692675	TSV	FOD	*cincta*
wBTFI026	02692676	TSV	FOD	*cincta*
wBTFI027	02692677	TSV	FOD	*cincta*
wBTFI028	02692678	TSV	FOD	*cincta*
wBTFI029	02692679	TSV	FOD	*cincta*
wBTFI030	02692680	TSV	FOD	*cincta*
wBTFI031	02692681	TSV	FOD	*cincta*
wBTFI032	02692682	TSV	FOD	*cincta*
wBTFI033	02692683	TSV	FOD	*cincta*
wBTFI034	02692684	TSV	FOD	*cincta*
wBTFI035	02692685	TSV	FOD	*cincta*
wBTFI036	02692686	TSV	FOD	*cincta*
wBTFI037	02692687	TSV	FOD	*cincta*
wBTFI038	02692688	TSV	FOD	*cincta*
wBTFI039	02692689	TSV	FOD	*cincta*
wBTFI040	02692690	TSV	FOD	*cincta*
wBTFI041	02692691	TSV	FOD	*cincta*
wBTFI042	02692692	TSV	FOD	*cincta*
wBTFI043	02692693	TSV	FOD	*cincta*
wBTFI044	02692694	TSV	FOD	*cincta*
wBTFI045	02692695	TSV	FOD	*cincta*
wBTFI046	02692696	TSV	FOD	*cincta*
wBTFI047	02692697	TSV	FOD	*cincta*
wBTFI048	02692698	TSV	FOD	*cincta*

§ This individual was not ringed due to a deformed leg, which wasn't suitable for ringing

SAMPLE ID	RING CODE‡	REGION	SITE	SUBSPECIES
wBTFI049	02692700	TSV	FOD	*cincta*
wBTFI050	02692701	TSV	FOD	*cincta*
wBTFI051	02692702	TSV	FOD	*cincta*
wBTFI052	02692703	TSV	FOD	*cincta*
wBTFI053	02692704	TSV	FOD	*cincta*
wBTFI054	not banded	TSV	FOD	*cincta*
wBTFI055	02692706	TSV	FOD	*cincta*
wBTFI056	02692707	TSV	FOD	*cincta*
wBTFI057	02692708	TSV	FOD	*cincta*
wBTFI058	02692709	TSV	FOD	*cincta*
wBTFI059	02692710	TSV	FOD	*cincta*
wBTFI060	02692713	TSV	FOD	*cincta*
wBTFI061	02692714	TSV	FOD	*cincta*
wBTFI062	02692715	TSV	FOD	*cincta*
wBTFI063	02692716	TSV	FOD	*cincta*
wBTFI064	02692721	TSV	CLW	*cincta*
wBTFI065	02692722	TSV	CLW	*cincta*
wBTFI066	02692723	TSV	CLW	*cincta*
wBTFI067	02692724	TSV	CLW	*cincta*
wBTFI068	02692725	TSV	CLW	*cincta*
wBTFI069	02692726	TSV	CLW	*cincta*
wBTFI070	02692727	TSV	CLW	*cincta*
wBTFI071	02692728	TSV	CLW	*cincta*
wBTFI072	02692729	TSV	CLW	*cincta*
wBTFI073	02692730	TSV	CLW	*cincta*
wBTFI074	02692732	TSV	DOD	*cincta*
wBTFI075	02692733	TSV	DOD	*cincta*
wBTFI076	02692735	TSV	CLW	*cincta*
wBTFI077	02692736	TSV	CLW	*cincta*
wBTFI078	02692737	TSV	CLW	*cincta*
wBTFI079	02692738	TSV	CLW	*cincta*
wBTFI080	02692739	TSV	CLW	*cincta*
wBTFI081	02692740	TSV	CLW	*cincta*
wBTFI082	02692741	TSV	CLW	*cincta*
wBTFI083	02692748	TSV	FOD	*cincta*
wBTFI084	02692749	TSV	FOD	*cincta*
wBTFI085	02692750	TSV	FOD	*cincta*
wBTFI086	02692751	TSV	FOD	*cincta*
wBTFI087	02692752	TSV	FOD	*cincta*
wBTFI088	02692753	TSV	FOD	*cincta*
wBTFI089	02692754	TSV	FOD	*cincta*
wBTFI090	02692755	TSV	FOD	*cincta*
wBTFI091	02692756	TSV	FOD	*cincta*
wBTFI092	02692757	CEQ	DES	*cincta*
wBTFI093	02692758	CEQ	DES	*cincta*
wBTFI094	02692759	CEQ	DES	*cincta*
wBTFI095	02692760	CEQ	DES	*cincta*

SAMPLE ID	RING CODE‡	REGION	SITE	SUBSPECIES
wBTFI096	02692762	CEQ	DES	*cincta*
wBTFI097	02692763	CEQ	DES	*cincta*
wBTFI098	02692764	CEQ	DES	*cincta*
wBTFI099	02692765	CEQ	DES	*cincta*
wBTFI100	02692766	CEQ	DES	*cincta*
wBTFI101	02692767	CEQ	DES	*cincta*
wBTFI102	02692768	CEQ	DES	*cincta*
wBTFI103	02692769	CEQ	DES	*cincta*
wBTFI104	02692770	CEQ	DES	*cincta*
wBTFI105	02692771	CEQ	DES	*cincta*
wBTFI106	02692772	CEQ	DES	*cincta*
wBTFI107	02692773	CEQ	DES	*cincta*
wBTFI108	02692774	CEQ	DES	*cincta*
wBTFI109	02692775	CEQ	DES	*cincta*
wBTFI110	02692776	CEQ	DES	*cincta*
wBTFI111	02692777	CEQ	DES	*cincta*
wBTFI112	02692778	CEQ	DES	*cincta*
wBTFI113	02692779	CEQ	DES	*cincta*
wBTFI114	02692780	CEQ	DES	*cincta*
wBTFI115	02692782	CEQ	DES	*cincta*
wBTFI116	02692783	CEQ	DES	*cincta*
wBTFI117	02692784	CEQ	DES	*cincta*
wBTFI118	02692785	CEQ	DES	*cincta*
wBTFI119	02692786	CEQ	DES	*cincta*
wBTFI120	02692787	CEQ	DES	*cincta*
wBTFI121	02692788	CEQ	DES	*cincta*
wBTFI122	02692789	CEQ	DES	*cincta*
wBTFI123	02692790	CEQ	DES	*cincta*
wBTFI124	02692791	CEQ	DES	*cincta*
wBTFI125	02692792	CEQ	DES	*cincta*
wBTFI126	02692793	CEQ	DES	*cincta*
wBTFI127	02692794	CEQ	DES	*cincta*
wBTFI128	02692795	CEQ	DES	*cincta*
wBTFI129	02692796	CEQ	DES	*cincta*
wBTFI130	02692797	CEQ	DES	*cincta*
wBTFI131	02692798	CEQ	DES	*cincta*
wBTFI132	02692799	CEQ	DES	*cincta*
wBTFI133	02692800	CEQ	DES	*cincta*
wBTFI134	02692801	CEQ	DES	*cincta*
wBTFI135	02692802	CEQ	DES	*cincta*
wBTFI136	02692813	FNQ	MAW	*atropygialis*
wBTFI137	02692815	FNQ	MAW	*atropygialis*
wBTFI138	02692816	FNQ	MAW	*atropygialis*
wBTFI139	02692817	FNQ	MAW	*atropygialis*
wBTFI140	02692818	FNQ	MAW	*atropygialis*
wBTFI141	02692819	FNQ	MAW	*atropygialis*
wBTFI142	02692820	FNQ	MAW	*atropygialis*

SAMPLE ID	RING CODE‡	REGION	SITE	SUBSPECIES
wBTFI143	02692821	FNQ	MAW	*atropygialis*
wBTFI144	02692822	FNQ	MAW	*atropygialis*
wBTFI145	02692823	FNQ	MAW	*atropygialis*
wBTFI146	02692831	FNQ	MAW	*atropygialis*
wBTFI147	02692833	FNQ	MAW	*atropygialis*
wBTFI148	02692834	FNQ	MAW	*atropygialis*
wBTFI149	02692835	FNQ	MAW	*atropygialis*
wBTFI150	02692838	FNQ	MAW	*atropygialis*
wBTFI151	02692841	FNQ	MAW	*atropygialis*
wBTFI152	02692849	TSV	ARC	*cincta*
wBTFI153	02692850	TSV	ARC	*cincta*
wBTFI154	02692851	TSV	ARC	*cincta*
wBTFI155	02692852	TSV	ARC	*cincta*
wBTFI156	02692853	TSV	ARC	*cincta*

APPENDIX F

Raw Data II: details of the Black-throated Finch samples acquired from the captivity used in the analysis

Origin codes:

SEQ: south-eastern Queensland

NSW: northern New South Wales

ROC: Rockhampton, central QLD

AYR: Ayr, northern Queensland

UNK: origin unknown

Source codes:

QLDa – QLDe: five breeders from Queensland denoted as a – e

NSWa – NSWe: five breeders from New South Wales denoted as a – e

SAMPLE ID	ORIGIN	SOURCE	SUBSPECIES
cBTFI001	SEQ	QLDa	*cincta*
cBTFI002	SEQ	QLDa	*cincta*
cBTFI003	SEQ	QLDa	*cincta*
cBTFI004	SEQ	QLDa	*cincta*
cBTFI005	SEQ	QLDa	*cincta*
cBTFI006	SEQ	QLDa	*cincta*
cBTFI007	SEQ	QLDa	*cincta*
cBTFI008	SEQ	QLDa	*cincta*
cBTFI009	SEQ	NSWa	*cincta*
cBTFI010	SEQ	NSWb	*cincta*
cBTFI011	SEQ	NSWb	*cincta*
cBTFI012	SEQ	NSWb	*cincta*
cBTFI013	SEQ	NSWa	*cincta*
cBTFI014	SEQ	NSWb	*cincta*
cBTFI015	SEQ	NSWb	*cincta*
cBTFI016	SEQ	NSWc	*cincta*
cBTFI017	SEQ	NSWb	*cincta*
cBTFI018	SEQ	NSWb	*cincta*

SAMPLE ID	ORIGIN	SOURCE	SUBSPECIES
cBTFI019	SEQ	NSWb	*cincta*
cBTFI020	NSW	NSWd	*cincta*
cBTFI021	NSW	NSWd	*cincta*
cBTFI022	SEQ	NSWd	*cincta*
cBTFI023	SEQ	NSWd	*cincta*
cBTFI024	UNK	NSWd	*cincta*
cBTFI025	SEQ	NSWd	*cincta*
cBTFI026	UNK	NSWd	*cincta*
cBTFI027	UNK	NSWd	*cincta*
cBTFI028	AYR	QLDb	*cincta*
cBTFI029	AYR	QLDb	*cincta*
cBTFI030	AYR	QLDb	*cincta*
cBTFI031	AYR	QLDb	*cincta*
cBTFI032	AYR	QLDb	*cincta*
cBTFI033	AYR	QLDb	*cincta*
cBTFI034	UNK	QLDc	*cincta*
cBTFI035	UNK	QLDc	*cincta*
cBTFI036	UNK	QLDc	*cincta*
cBTFI037	UNK	QLDc	*cincta*
cBTFI038	UNK	QLDc	*cincta*
cBTFI039	UNK	QLDc	*cincta*
cBTFI040	UNK	QLDc	*cincta*
cBTFI041	UNK	QLDc	*cincta*
cBTFI042	UNK	QLDc	*cincta*
cBTFI043	UNK	QLDc	*cincta*
cBTFI044	UNK	QLDc	*cincta*
cBTFI045	UNK	QLDc	*cincta*
cBTFI046	UNK	QLDc	*cincta*
cBTFI047	UNK	QLDc	*cincta*
cBTFI048	UNK	QLDc	*cincta*
cBTFI049	UNK	QLDc	*cincta*
cBTFI050	UNK	QLDd	*unknown*[**]
cBTFI051	UNK	QLDd	*unknown*
cBTFI052	UNK	QLDd	*unknown*
cBTFI053	UNK	QLDd	*unknown*
cBTFI054	UNK	QLDd	*unknown*
cBTFI055	UNK	QLDd	*unknown*
cBTFI056	UNK	QLDe	*unknown*
cBTFI057	UNK	QLDe	*unknown*
cBTFI058	UNK	QLDe	*unknown*
cBTFI059	UNK	QLDe	*unknown*
cBTFI060	UNK	NSWc	*cincta*

[**] These individuals showed mixed rump colours and they were the offspring of both subspecies.

SAMPLE ID	ORIGIN	SOURCE	SUBSPECIES
cBTFI061	UNK	NSWc	*cincta*
cBTFI062	UNK	NSWc	*cincta*
cBTFI063	UNK	NSWc	*cincta*
cBTFI064	UNK	NSWc	*cincta*
cBTFI065	UNK	NSWc	*cincta*
cBTFI066	UNK	NSWc	*cincta*
cBTFI067	UNK	NSWc	*cincta*
cBTFI068	UNK	NSWc	*cincta*
cBTFI069	UNK	NSWc	*cincta*
cBTFI070	UNK	NSWc	*cincta*
cBTFI071	UNK	NSWc	*cincta*
cBTFI072	UNK	NSWc	*cincta*
cBTFI073	UNK	NSWc	*cincta*
cBTFI074	UNK	NSWc	*cincta*
cBTFI075	UNK	NSWc	*cincta*
cBTFI076	UNK	NSWc	*cincta*
cBTFI077	UNK	NSWc	*cincta*
cBTFI078	UNK	NSWc	*cincta*
cBTFI079	UNK	NSWc	*cincta*
cBTFI080	ROC	NSWe	*cincta*
cBTFI081	ROC	NSWe	*cincta*
cBTFI082	ROC	NSWe	*cincta*
cBTFI083	ROC	NSWe	*cincta*
cBTFI084	ROC	NSWe	*cincta*
cBTFI085	ROC	NSWe	*cincta*
cBTFI086	ROC	NSWe	*cincta*
cBTFI087	ROC	NSWe	*cincta*
cBTFI088	ROC	NSWe	*cincta*
cBTFI089	ROC	NSWe	*cincta*
cBTFI090	ROC	NSWe	*cincta*
cBTFI091	ROC	NSWe	*cincta*
cBTFI092	ROC	NSWe	*cincta*
cBTFI093	ROC	NSWe	*cincta*
cBTFI094	ROC	NSWa	*cincta*
cBTFI095	ROC	NSWa	*cincta*
cBTFI096	ROC	NSWa	*cincta*
cBTFI097	ROC	NSWa	*cincta*