# Phylogeography, demography and recruitment in a widespread iconic Australian bird, Gymnorhina tibicen


## Author

McIntyre, Nathan D



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# PHYLOGEOGRAPHY, DEMOGRAPHY AND RECRUITMENT IN A WIDESPREAD ICONIC AUSTRALIAN BIRD, *GYMNORHINA TIBICEN*

Nathan McIntyre B.Sc (Hons)

School of Environmental and Physical Sciences

Griffith University

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# SYNOPSIS

With the advent of efficient and affordable sequencing technology, it has become increasingly viable to generate whole or reduced-representation genome sequence data for non-model organisms. The surfeit of genetic information contained in these datasets can be used in a wide range of studies. These include the assessment of genetic structure and diversity among contemporary populations, the investigation of past divergence events and demographic history, and the identification of variants or loci involved in adaptation. Not only do these insights afford us a greater understanding of the evolutionary history and likely future trajectory of a species, but they also allow us to inform conservation and wildlife management programs by identifying at-risk subpopulations and gauge the ability of a species to respond to environmental changes resulting from climate change or habitat loss.

In this thesis, I investigate aspects of the population genetic structure and diversity of *Gymnorhina tibicen*, the Australian magpie, as well as phylogeographic relationships within the species and the impact that weather conditions have on the species' ability to recruit new individuals. *G. tibicen* is an iconic member of the Australian avifauna, a medium-sized generalist passerine known for its characteristic 'fluting' call, stark black and white plumage, and aggressively territorial behaviour. Its distribution covers most of mainland Australia and Tasmania, as well as parts of Papua New Guinea, and has been recorded as an invasive species in New Zealand.

Currently, the phylogeography & demography of the Australian magpie has been examined using a single mitochondrial gene & a small number of microsatellite loci. In Chapter 2, I use

genomic data to explore the structure, diversity, and biogeography of southern populations of *Gymnorhina tibicen.* I utilize restriction site-associated (RAD) sequencing to generate a dataset consisting of over 29,000 single nucleotide polymorphisms (SNPs) distributed over more than 15,000 loci. I detail the process of preparing the RADseq dataset, including the testing of parameters that influence the assembly of loci from raw reads. I then use the dataset to examine several hypotheses: that due to the influence of major biogeographic barriers across mainland Australia and Tasmania, reduced-representation genomic data would reveal clear structuring between eastern, western and Tasmanian populations of the Australian magpie, that the western group would be more structured and exhibit a more stable demographic history while the eastern would show signs of population expansion due to the more widespread temperate climate. I use ten populations of *G. tibicen* distributed across south-eastern and south-western Australia, including two populations from Tasmania. Tests for genetic clustering and fine-scale genetic structure among populations revealed a clear distinction between eastern, western and Tasmanian populations, with Tasmanian samples more closely related to the eastern mainland than western. Western populations also appear more differentiated from one another than those in the eastern cluster, supporting my initial hypothesis. Demographic analyses also suggested a greater rate of expansion in eastern mainland Australia, compared to the relatively stable western mainland.

Previous phylogeographic studies of *Gymnorhina tibicen* made use of a single mitochondrial gene, but recent advances in sequencing technology make it possible to investigate phylogeographic relationships across entire mitogenomes to verify earlier results and increase the genetic resolution at which inferences are made. In Chapter 3, my goal was to assemble whole mitochondrial genomes from specimens of *G. tibicen* across its distribution, and compare phylogenetic trees constructed from individual genes and complete + near-complete

mitogenomes. Previously, a single attempt had been made to assemble a complete *G. tibicen* mitogenome from a museum eggshell specimen, which produced a near-complete sequence minus a repetitive area of the control region. In my study, five complete mitogenomes were assembled, with the remainder assembled into partial but near-complete sequences. Analysis of the mitogenomes assembled here revealed the same broad patterns of divergence between western and eastern halves of the Australian mainland, and closer relationship of the Tasmanian cluster to the eastern group than the western. This study incorporates new sampling locations into our understanding of the phylogeography of *G. tibicen*, and most notably suggests that the 'eastern' group may extend further west across the arid interior than previously identified. The results here also demonstrate the efficacy and improved resolution of phylogeographic analyses when whole mitogenomes are used in lieu of single genes.

Long-term ecological datasets are often difficult to produce, but are invaluable when estimating the impact of changing environmental conditions on native wildlife. In Chapter 4, I analyse recruitment data collected over 25 years as part of a long-term territory monitoring study. Birds from over 200 territories were banded and monitored twice yearly, with a range of observations and biological samples taken. I have taken one aspect of the collected data, the number of new juveniles observed in each territory annually, and investigated how recruitment is influenced by variation in local weather conditions over the course of the year preceding the breeding season. I conclude that *Gymnorhina tibicen* appears relatively robust in the face of annual variation in weather and adverse conditions such as drought, though lower levels of precipitation in the leadup to the breeding season are linked to lower numbers of juveniles recruited per territory. I tentatively suggest that this is result of reduced invertebrate abundance and therefore reduced foraging opportunities, impacting the reproductive health of individuals.

Overall, genomic studies of the Australian magpie still have much to offer, particularly in the investigation of demographic history and the identification of regions under selection. Principal recommendations for building on the questions covered in this study are a more thorough sampling regime, both in total number of samples and their spatial resolution across *G. tibicen*'s distribution.

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Finally, I would like to dedicate this thesis in memory of my grandmother, Margaret Tait.

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# STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma at any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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Nathan McIntyre

# CHAPTER 1: GENERAL INTRODUCTION

### *1.1.1 Forces influencing genetic variation*

The genetic variation among individuals and populations is subject to evolutionary processes, the most important being mutation, genetic drift, gene flow and natural selection. Understanding these mechanisms, and how they influence the frequency of alleles in populations, is integral to population genetic and genomic studies, especially those that aim to investigate the demographic histories of species (Black et al., 2001, Gong et al., 2015, Tsuda et al., 2015, Vera-Escalona et al., 2015).

New alleles ultimately arise from mutations, where the nucleotide sequence of a gene is altered permanently, increasing genetic variation. Mutations may be neutral, or they may alter the gene in a manner that benefits or has a deleterious effect on the individual bearing the mutation. If a new allele increases the reproductive fitness of the individual that bears it, its frequency in the population will increase as its carrier competes successfully for resources and produces more offspring than competitors. Conversely, if an allele has a deleterious effect, reproductive success is less likely and its frequency in the population will decrease. Selection is the process by which genetic variants that confer reproductive advantages persist into subsequent generations (Darwin, 1859, Fisher, 1930).

Alleles being sampled randomly from one generation to the next impacts the frequency at which they occur. In small populations, the stochastic process can result in lower frequency genetic variants being completely lost, even if they were beneficial to the individual, and more common alleles can become fixed within a population (Wright, 1931, Lande, 1976,

Ellstrand and Elam, 1993). The rate of drift is dependent on effective population size ($N_e$), the size of the ideal population (N) that will result in the same amount of genetic drift as the population being examined. $N_e$ can be thought of as the number of individuals that successfully contribute offspring to the next generation (Allendorf and Luikart, 2009), and is of particular concern to conservation studies (Hare et al., 2011, Cammen et al., 2018, Antao et al., 2011). The consequences of these demographic shifts can be significant. Population bottlenecks and low $N_e$ over a prolonged period can lead to lowered heterozygosity and permit deleterious alleles to persist in a population where they would normally be outcompeted. In larger populations, the influence of genetic drift is greatly reduced.

Where diversity is lost due to drift, equilibrium can be maintained through the introduction of new alleles via mutation. Alternatively, new alleles can be introduced via migration of new individuals from one population to another. The term 'gene flow' is used to describe the process by which populations exchange genes. Gene flow can be a product of the migration of individuals among populations, but also a product of the mechanical transfer of eggs, seeds or gametes via wind, water or animal-aided dispersal. Regardless of the mechanism, gene flow can lead to the homogenization of allele frequencies across populations in the absence of differential selective pressures (Hartl et al., 1997).

Together, these four mechanisms are responsible for changes in the frequency of alleles in a population over generations. Mutation and gene flow introduce new alleles into a population. Natural selection and genetic drift phase out alleles based on the production of unfavourable phenotypes for survival or unsustainably low frequency, respectively. The relative influence

of these processes determines whether the relative frequencies of alleles in that population remain stable, decline or increase over time.

In many population genetic methods, there is an assumption that the population in question can be approximated by an idealised Wright-Fisher model (Hartl et al., 1997), and therefore there are no genetic or behavioural influences on mating. Panmixia describes the situation where mating of individuals in the same population occurs randomly, regardless of distance or individual behaviour. However, panmixia is seldom the case in naturally occurring populations. Non-random mating is a result of life history traits such as philopatry, in which individuals return to or remain in an area, ecological factors such as habitat preference, or the presence of environmental barriers that limit dispersal, such as mountains, rivers or inhospitable regions. As a result, groups of individuals within the same geographic region tend to be more closely related to each other than to groups in different regions, creating spatial patterns of genetic differentiation, or genetic structure. Eventually, this leads to genetically distinct populations (Hartl et al., 1997, Glor and Warren, 2011).

### *1.1.2 Genetics to genomics: Molecular techniques for the study of native wildlife*

Mitochondrial DNA was one of the earliest tools used in studies of genetic variation, due to the relative simplicity of smaller organellar genomes (Avise, 2012). With the advent of Polymerase Chain Replication (PCR) (Saiki et al., 1985, Saiki et al., 1988), regions of interest could be selectively amplified for easier analysis and visualisation, and markers such as amplified fragment length polymorphisms (AFLPs, Vos et al. (1995)) were introduced for use in plant and microbial studies, and remained the mainstay for genome-wide analyses until genome-wide SNP datasets and sequencing techniques were developed (Luikart et al., 2003).

Microsatellites have also been widely used as genetic markers due to the ability to directly score heterozygotes and the repeatable nature of the method (Weber and May, 1989). Also known as simple sequence repeats (SSRs) and short tandem repeats (STRs), microsatellites are genomic regions that feature tandem repeats of a simple motif of 1-4 nucleotides. Microsatellite loci tend to be highly variable, often heterozygous, and are assumed to be selectively neutral. While the sequence itself is not directly observed, the size of an allele (read: the total number of repeats) can be detected using gel electrophoresis or fragment size analysis. They have been demonstrated to be informative when detecting population bottlenecks and population size changes at contemporary scales, given a large sample size (Antao et al., 2011, Tallmon et al., 2010). However, accuracy is reduced when dealing with large populations. Adding more microsatellite loci to a dataset increases the power of analyses to detect demographic changes, but in most cases it is impractical to develop hundreds of microsatellite markers that would only simulate relatively few (100-1000) Single Nucleotide Polymorphisms (Hollenbeck et al., 2016, Antao et al., 2011). Additionally, not all microsatellite markers meet the assumption of neutrality, as some studies have detected selection on microsatellite loci (Chhatre and Rajora, 2014, Edelist et al., 2006) and associated microsatellite length with diseases e.g. Crohn's disease in humans (Hugot et al., 2001). Larger genomic data sets offer increased power for estimating accurate changes in population size.

Genomic-scale data can be used to make powerful inferences about the demographic history of populations. While microsatellites and mitochondrial markers allow the detection of bottlenecks and other changes in population size using methods such as Approximate Bayesian Computation, the statistical power of genomic datasets when used with similar methods is far greater (Luikart et al., 2003, Pool et al., 2010). Even reduced-representation

genomic data, which might cover less than 5% of the actual genome of an organism, is a powerful tool for biogeographic inference, as tens of thousands of SNP loci are genotyped across large numbers of individuals. Patterns and trends derived from genomic data can be used to inform approaches to numerous contemporary issues facing native wildlife , aiding studies of invasion dynamics, climate-driven population shifts (Prates et al., 2016), and the dynamics of glacial refugia (Kopuchian et al., 2016), and models can be tested across multiple populations, comparing the connectivity and demography of multiple species that occupy similar distributions (Oswald et al., 2017).

### *1.1.3 Biogeographic history of Australia*

Many plant and animal lineages in Australia underwent diversification during the Pliocene epoch (5.3 to 2.6 million years ago), during which environmental conditions in Australia were warmer and wetter on average than the current conditions of the Anthropocene. As the Pliocene gave way to the Pleistocene, major climatic oscillations began to occur as the earth fluctuated between glacial periods and interglacial periods (Berger, 1984). The amplitude of these oscillations peaked over the last 0.4 Myr, exceeding thresholds for stabilised vegetation during glacial maxima and resulting in widespread erosion. These environmental changes had a major influence on the composition and distribution of species worldwide (Avise and Walker, 1998, Provan and Bennett, 2008). Climatic extremes, from glacial to interglacial maxima, resulted in extreme temporal and spatial variability of late Quarternary environments, and the Australian continent became progressively drier (Byrne, 2008). By the last glacial maximum (LGM), 21000 ± 2000 years ago, the continent was experiencing widespread and locally extreme aridity. Arid-zone species and lineages that arose during the Pliocene underwent major intraspecific diversification during the Pleistocene, persisting during the dramatic climatic and environmental changes (Byrne et al., 2008, McLeish et al., 2007, Chapple et al., 2004, Fujita et al., 2010).

Climatic oscillations during the Pleistocene have been identified as playing a major role in the shaping of contemporary biodiversity in Australia. The dominant assumption in biogeography literature is that during glacial maxima (GM), species undergo range contraction and persist in one or few refugia, and during interglacial periods they experience range expansions, secondary contact and gene flow between refugia (Hewitt, 2004). This GM contraction model was developed using examples of temperate species in Northern

hemisphere environments, and while it is widely accepted, it has also recently been acknowledged as not being universally applicable. Cold-adapted taxa in the northern hemisphere have been suggested to follow an inverse model of GM expansion, undergoing range expansion and gene flow during GM and retreating to cooler, higher latitudes during warmer, wetter interglacials (Galbreath et al., 2009). The response of arid-adapted species to increased aridity during GM may be analogous to the cold-adapted biota of the Northern Hemisphere, and some studies and reviews of Australian arid-tolerant biota have begun to challenge the default GM contraction model (Kearns et al., 2014, Byrne, 2008, Byrne et al., 2008, Joseph and Omland, 2009).

Several environmental and geographic barriers have been identified as influential in shaping the biogeography of southern Australia. At the geographical centre, the limestone-based Nullarbor Plain spans approximately 200,000km$^2$ (Webb and James, 2006), and has been hypothesised to be critical in the formation of the entire region's biogeography (Crisp and Cook, 2007). The region is fringed by belts of semi-arid vegetation systems, most commonly mallee. The south-western and south-eastern regions of mainland Australia are mesic forest zones which possess some of the continent's highest levels of endemism (Byrne et al., 2011), and contain numerous pairs of mesic sister-species. The southwestern region of Australia is considered one of 34 global biodiversity hotspots (Mittermeier et al., 1999), with particularly high levels of avian endemism and plant species diversity. In addition to the Nullarbor, the Eyrean, Swan and Bassian barriers have all been hypothesised to be critical in the formation of contemporary patterns of diversity, by vicariance and dispersal across them (Ford, 1987, Keast, 1961, Schodde and Mason, 1999).

Similar patterns of divergence have been observed in a wide variety of taxa. Past reviews of phylogeographic work on avian species (Dolman and Joseph, 2015, Joseph and Omland, 2009) have identified the importance of the Eyrean barrier (some 1000km east of the Nullarbor) in particular. Other vertebrate studies that tested hypotheses regarding the biogeographic role of southern Australian barriers included reptiles (Dubey and Shine, 2010), amphibians (Symula et al., 2008) and mammals (Neaves et al., 2009, Cooper, 2003). These studies all have identified strong genetic disjunctions between the eastern and western reaches of their distributions. Further south, the timing of the repeated formation and erosion of the isthmus joining Tasmania to the mainland has been inferred to be a significant barrier for dispersal in multiple species (McKinnon et al., 2004, Dawson, 2005). While these and other barriers are frequently invoked as the underlying cause of observed disjunctions in species distributions, further study is needed to cement these putative biogeographic barriers and define their roles in speciation, divergence and community assembly (Glor and Warren, 2011).

### *1.1.4 Study Species: The Australian magpie (Gymnorhina tibicen)*

The Australian magpie (*Gymnorhina tibicen*) is a medium-sized passerine bird, native to Australia and southern New Guinea. It exhibits marked morphological variation across its distribution, and is currently recognised as up to eight subspecies on the basis of size, bill length, wingspan and plumage pattern (Schodde and Mason, 1999). For most of the species' history, it has been placed within the monotypic *Gymnorhina* due to its distinctive morphology and behaviour. However, recent studies have prompted debate as to whether the Australian magpie should be folded into its sister genus of *Cracticus,* the Australian Butcherbirds (Christidis et al., 2008). This was reinforced by a recent phylogenetic study, which showed that Australian magpies are likely sister to black butcherbirds, and nested within the clade of butcherbirds (Kearns et al., 2013). While this revised taxonomic placement has been acknowledged as 'nomenclaturally valid' (Cake et al., 2018), it has been argued that the comparatively robust, terrestrial Australian magpie should still be recognised as *Gymnorhina*, as synonymising it with *Cracticus* would fail to properly capture the evolutionary history and adaptive diversity across the group (Cake et al., 2018). For the sake of consistency, in this document, the Australian magpie will be referred to as *Gymnorhina tibicen* throughout, although the taxonomic debate may continue.

Plio-Pleistocene changes in climate have played a significant role in the subsequent evolutionary history of *G. tibicen* and its close relatives. Specifically, aridification in central and western Australia during the Pleistocene has resulted in a pattern of east-west genetic divergence in *Cracticus* species (Kearns et al., 2011). In *G. tibicen*, study of the mitochondrial DNA control region revealed that eastern and western populations form monophyletic clades, with close but nonoverlapping distributions (Toon et al., 2017). The

timing of this divergence has been estimated to be around 36000 years ago and is associated with the Nullarbor and Carpentarian barriers in southern and northern Australia, respectively (Toon et al., 2007). A second more recent divergence was identified between Tasmanian and mainland populations, approximately 16000 years ago (Toon et al., 2007).

*Differences in magpie social structure and behaviour*

The eight subspecies of *Gymnorhina tibicen* can be grouped based on back colour: Four black-backed (BB) subspecies are distributed across northern Australia, three white-backed (WB) subspecies situated in south-eastern continental Australia and Tasmania, and a single varied subform of white-backed magpies is restricted to south-west Australia (*G. tibicen dorsalis*). The third plumage group exhibits stronger sexual dimorphism than WB and BB groups, with males that resemble white-backed individuals, while females have black feathers that are edged or scalloped in white. All plumage forms seem to interbreed where their distributions overlap, with regions of north-south intergrade spanning hundreds of kilometres (Hughes, 1982, Burton and Martin, 1976). Intermediate morphs exhibit varying degrees of black and white plumage. In southern Australia, female back colour appears to vary along a cline between the varied and white-backed morphs. Most commonly, members of the eight subspecies of *Gymnorhina tibicen* live in pairs or groups on permanent all-purpose territories. Some populations breed cooperatively, where both biological parents and additional group members care for offspring, while others breed plurally, with multiple females nesting and mated with the same or different males (Hughes et al., 2003).

Unlike many territorial bird species, in magpies the female is more philopatric, remaining in the natal territory while males disperse to other territories more often (Veltman and Carrick,

1990). Higher levels of natal philopatry appear to exist in southwestern populations than white-backed or black-backed, with mtDNA suggesting that extremely high gene flow occurs among eastern populations (Baker et al., 2000). The size of group territories varies across the continent, with southwestern populations having the largest group sizes (3-26), followed by Tasmanian populations (3-15) (Hughes and Mather, 1991). There is also a much higher degree of extra-group paternity (EGP) in southwestern populations than white-backed magpie populations. In a prior study, eastern white-backed *G. t. tyrannica* populations were found to have EGP rates of approximately 44%, while south-western *G. t. dorsalis* populations exhibited rates of up to 82%, one of the highest observed in a Passerine species (Durrant and Hughes, 2005, Hughes et al., 2003).

### *1.1.5 Project Aims*

The primary aims of my thesis were to investigate contemporary and historical patterns of genetic connectivity and demography in populations of *Gymnorhina tibicen* across the species' distribution, with an initial focus on areas in south west and south eastern Australia speculated to serve as refugia for the species during glacial maxima. A recent study had demonstrated the viability of generating reduced-representation genomic data from the species for a single site (Prince, 2015), and I aimed to use the same molecular techniques on a broader geographic scale. The main hypotheses I planned to test in this project were that the influence of major biogeographic barriers such as the Nullarbor and Bass Strait would produce similar patterns in reduced representation genomic data as the small number of genes in previous studies: mainland populations of *G. tibicen* in the east and west of the continent would be genetically distinct from one another, and Tasmanian populations would be distinct from those on the mainland. I also anticipated that differing climate history between the

eastern and western mainland would produce contrasting demographic signals following the Last Glacial Maximum. To test this hypothesis, I compare genetic diversity, connectivity, and demography between eastern and western populations, anticipating that eastern populations would show fewer signs of genetic structuring among populations and signatures of population expansion thanks to the abundance of suitable habitat, relative to more structured and slower-growing western populations.

My second project aimed to produce multiple circularised genomes for the mitochondria of *G. tibicen* specimens across their range. I also aimed to investigate similar biogeographic patterns as in my first chapter, comparing the signals derived from individual mitochondrial genes with those of complete mitogenomes. I hypothesised that the same broad groupings would be apparent regardless of the specific genes used.

During my candidature, an opportunity also arose to analyse elements of a long-term ecological dataset collected over 25 years at a field site in rural Victoria (Chapter 5). With this study, my aims were to investigate the impact of variation in local weather conditions on the recruitment of new juveniles into established territories of *Gymnorhina tibicen*. I hypothesised that cooler summers and higher annual rainfall would lead to increased recruitment for magpie territories, as they would experience lower levels of thermoregulatory stress and improved foraging success. As the study area was predominately pastoral farmland, I expected the abundance of artificial surface water (i.e. dams and troughs) would mitigate the impacts of severe heat or drought on recruitment.

# CHAPTER 2: POPULATION GENOMICS OF *GYMNORHINA TIBICEN* IN SOUTHERN AUSTRALIA

## 2.1 Abstract

For many naturally occurring species, the study of patterns of genetic diversity and demographic history has arrived at next-generation sequencing (NGS) as the approach of choice. As whole genome and reduced-representation sequencing technology increases in sophistication and becomes more cost effective, it becomes more viable to investigate non-model organisms using thousands, even hundreds of thousands, of single nucleotide polymorphisms distributed throughout the genome. In this chapter, I have generated a reduced-representation genomic dataset for the Australian magpie (*Gymnorhina tibicen)* using restriction site-associated DNA sequencing (RADseq) methods. I test a range of parameters used in the assembly of loci from raw reads, and produce a dataset of over 29 thousand single nucleotide polymorphisms (SNPs), distributed across more than 15,000 variable loci. My aim was to make inferences regarding patterns of contemporary and historical connectivity and demography among populations of *G. tibicen*, with much higher genetic resolution than has previously been used. In my results, I have characterised the genetic diversity within and among my study populations, identifying eastern, western and Tasmanian clusters, with the Tasmanian more closely related to populations on the eastern mainland. Greater levels of interrelatedness were observed among eastern populations than western or Tasmanian. Extended Bayesian Skyline analyses of each cluster suggests a history of continuous population expansion in eastern *G. tibicen* populations, while both western mainland and Tasmanian populations show signs of a historical increase in population size, followed by rapid decrease and stabilisation towards the present.

## 2.2 Introduction

### *2.2.1 Inferences from patterns of genetic variation*

By investigating spatial patterns of genetic variation, it is possible to make inferences about the role that the environment plays in genetic divergences (Bjorklund et al., 2013, D'Amen et al., 2013), dispersal patterns, migration rates, and fluctuations in density and effective population size ($N_e$) of an organism. These responses may be linked to environmental variables and have broader applications for resource management and conservation efforts. This is particularly relevant in the face of climate change and human activity, both of which have an impact on biodiversity (Amar et al., 2014, D'Amen et al., 2013, Thuiller, 2007, Barnosky et al., 2004, Nelson et al., 2006). Major climatic events leave signatures in the DNA of populations. For example, genetic divergence among populations might reflect fragmentation of habitat due to glacial cycles (Taberlet et al., 1998, Mayr and O'Hara, 1986, Moritz et al., 2000) or the formation and erosion of land bridges due to changing sea levels (Kearns et al., 2011, Stange et al., 2018), and more or less suitable climatic conditions for a species may lead to sudden bottlenecks or population expansions (Tajima, 1989a). Temporal changes in genetic variation, however, are more difficult to study, as population-level sampling of time series genetic data is hard to obtain outside of study species with short generation times (Wang, 2005, Luikart et al., 2010).

The inference of temporal changes in genetic variation has been key to numerous studies (Park et al., 2015, Spurgin et al., 2014, Ramakrishnan and Hadly, 2009), and has a range of purposes. Contemporary genetic data has been used to infer population declines (Rodríguez-Zárate et al., 2013, Salmona et al., 2012), investigate species responses to climate change (Serra-Varela et al., 2015, Soltis et al., 2006), and examine ecological factors associated with

570 population divergence and speciation (Richards et al., 2007). Ancient DNA from sub-fossil specimens can also be used to time past divergence events, infer demographic changes and historical levels of genetic variation, as in studies of King and Adelie Penguins in Antarctica that have used frozen tissue preserved for thousands of years (Ritchie et al., 2004, Heupink Tim et al., 2012), or a study of extinct lion subspecies from northern Africa, India and the
575 Middle East which used museum fossil collections (Barnett et al., 2014). At the most basic level, the demographic history of populations can be hinted at by describing genetic variation within and among populations and observing patterns, but more sophisticated demographic inferences can be derived from model-based coalescent analyses.

580 Early demographic studies used the distribution of pairwise differences among DNA sequences to detect past changes in population size (Rogers and Harpending, 1992, Watterson, 1975, Tajima, 1983, Slatkin and Hudson, 1991). Sudden population size increases can be observed in the form of a unimodal distribution of pairwise genetic differences, with the timing being inferred from the position of the peak (Rogers and Harpending, 1992).
585 Statistical tests such as Fu's *Fs* are also capable of detecting population growth using haplotype distributions. Another measure of genetic variability, the number of segregating sites (nucleotide regions that exhibit polymorphism within a sample of DNA sequences) within a population, can also be used to infer whether a population has grown in size (Fu, 1995, Bertorelle and Slatkin, 1995, Tajima, 1989a).

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Population bottlenecks, a contrasting phenomenon in which a sharp decline in population size occurs due to environmental factors (such as disasters, disease, predation etc.), also leave genetic signatures. Early tests for bottlenecks focused on the detection of departures from

expectations under mutation-drift equilibrium. Allele frequency spectra (AFS), the distribution of allele frequencies for a given set of loci, can be plotted to obtain a visual illustration of the extent to which populations deviate from expectations under mutation-drift equilibrium. In more recent times, analyses have been developed to infer demographic history from AFS data (Gutenkunst et al., 2009, Nei et al., 1976). Positive values of Tajima's *D*, a classic test for mutation-drift equilibrium intended to detect selection, can be used to infer long-term changes in effective population size due to a lesser reduction in nucleotide diversity than in segregating sites (Tajima, 1989b). Other commonly used approaches include M-ratio (ratio of the number of alleles to the range in allele size) and heterozygosity excess tests based on multilocus microsatellite genotypes (Garza and Williamson, 2001, Cornuet and Luikart, 1996). Microsatellite methods like these have been used extensively (Peery et al., 2012).

### *2.2.2 Coalescent Theory*

There has been an increase in interest towards the reconstruction of the past demography of populations, as well as the estimation of contemporary demographic parameters, as DNA sequencing technology has developed (Avise, 2000, Emerson et al., 2001). Evolutionary biology, phylogeography and conservation biology have all flourished as a result of these technological advances, aided by the introduction of coalescent theory (Kingman, 1982, Hudson, 1990).

Prior to the development of coalescent theory, the Wright-Fisher model was the main tool used to estimate the probability of fixation of new mutations and the time to fixation/loss under different conditions (Avise, 2012). While useful when trying to predict future changes

in genetic diversity, the WF model was less useful for historical inference. The coalescent theory is a population model that aims to model the genealogy of a group of individuals backwards in time, tracing lineages and keeping track of convergences until the Most Recent Common Ancestor (MRCA) is found. Early iterations of coalescent theory assumed a lack of selection and recombination, but as the model developed it has been expanded to incorporate a variety of evolutionary and demographic processes, allowing it to account for phenomena such as selfing (Nordborg, 1997) and population structure with migration (Wakeley, 2009).

Coalescent theory allows us to make inferences about population parameters such as *Ne*, as well as directional shifts in past population sizes, which are reflected by genealogy. The probability that any two DNA sequences drawn from a population coalesce at a given point in time is a function of the population's size (Kingman, 1982). Two DNA sequences taken randomly from a small population are more likely to have a recent coalescence (fewer substitutional differences separating the two sequences) than two sequences from a larger population. The signatures left in the patterns of DNA substitutions can therefore reflect the direction and timing of demographic changes (Emerson et al., 2001). It should be kept in mind that two independently evolving genes may have vastly different genealogies, and so provided a single population is being studied, and panmixia and a constant population size are assumed, increasing the number of loci in an analysis can be a viable alternative to sequencing more individuals (Schopen et al., 2008, Heled and Drummond, 2008).

### *2.2.3 RAD Sequencing*

Restriction-site Associated DNA sequencing (RADseq) is one method for obtaining genomic data from a reduced subset of the genome (Andrews et al., 2016, Davey and Blaxter, 2011,

Miller et al., 2007). The technology can generate thousands to tens of thousands of orthologous loci – regions that encode for the same protein. In this case, the region surrounds a restriction site, a specific sequence which is recognised by restriction enzymes that cleave DNA. This approach produces fragments of a particular size, usually 150-500bp, with the enzyme cut site on one end.

Protocols for preparing RAD tag libraries for sequencing vary, but a general overview is as follows. Genomic DNA of sufficient quality and concentration is digested with a restriction enzyme (eg. *SbfI* or *EcoRI*, to name two commonly used enzymes), and an adapter is ligated to the ends of the fragments which contains primer sites for sequencing and a nucleotide barcode unique to each sample (in the set of samples being sequenced). The fragments with the 'P1' adapters attached are then pooled together and randomly sheared. The sheared samples are run in a gel electrophoresis rig, and fragments within the desired size range are excised from the gel and purified. A second P2 adapter is then ligated to the other end of the DNA fragments that only allows the reverse amplification primer to bind if the P1 adapter is also ligated to the tag. RAD tags with a P1 adapter are PCR amplified before being sequenced (Baird et al., 2008). A variant protocol for RAD library preparation known as double digest (ddRAD) RAD library preparation uses a second restriction enzyme and only sequences fragments within a certain size range that are flanked by both enzyme cut sites, further reducing the complexity of the library (Peterson et al., 2012). While ddRAD requires two restriction enzymes, the protocol is less time consuming, does not require use of a sonicator to randomly shear the DNA into fragments, and has fewer cleanup steps in which DNA could be lost. Potential caveats when opting to use a ddRAD library is the increased likelihood of allele dropout and detection of PCR duplicates being more difficult (Arnold et

al., 2013). Ultimately, the selection of single or ddRAD sequencing depends on the desired level of complexity in the dataset and the available laboratory equipment.

*Applications of RADseq in population genomics*

Restriction-site Associated DNA sequence data has been used extensively in population genomic studies with a broad variety of purposes. It has been used in studies of introgression, hybridization and genomic clines (Gompert et al., 2017, Stolting et al., 2013, Rahman et al., 2018), scanning the genome for regions under selection (Van Wyngaarden et al., 2017, Laporte et al., 2016, Hohenlohe et al., 2010), and the inference of demographic history from contemporary samples (Harvey and Brumfield, 2015, Trucchi et al., 2014, Heupink Tim et al., 2012). Phylogenetic studies have also benefited, with the advent of genomic-scale analyses for clarifying evolutionary relationships between groups (Sally et al., 2016, McCormack et al., 2013, Razkin et al., 2016, Pante et al., 2014). All of these applications assume that most of the genome being studied evolves neutrally, and that therefore the RAD tags produced represent a large quantity of randomly selected neutral sequence variation. This assumption is important in studies of population structure, demographic history and evolutionary relationships, but RAD tags cannot always be assumed to be neutral. As restriction enzyme-associated sites are scattered semi-randomly throughout the entire genome, some may reside in coding regions.

*Limitations of RADseq*

There are some properties of RADseq data that can lead to allele dropout and missing data. Mutations at restriction sites and undersequencing can lead to missing genotypes for loci, which in turn can be misinterpreted, resulting in heterozygous loci being identified as homozygous. These issues can lead to the estimated allele frequency of a dataset being skewed (Arnold et al., 2013), and misrepresents the site frequency spectrum (SFS) (Shafer et al., 2017). There are numerous studies that highlight the downstream effect of missing alleles on analysis (Gautier et al., 2013, Huang and Knowles, 2014, Arnold et al., 2013). To mitigate this, it is important to ensure loci with incomplete sampling are identified and removed, and differences in coverage among sites must be investigated in case they are the product of missing data (Arnold et al., 2013). Additional sources of bias include sequencing errors, coverage cutoffs and alignment errors, but these are widely known and are almost expected in sequencing projects (Huang and Knowles, 2014, Pool et al., 2010, Rokas and Abbot, 2009). The sampling design employed in studies of demographic history using RADseq must be considered carefully as well, as undetected population structure can produce misleading demographic signals (Mazet et al., 2016, Heller et al., 2013). If sampled individuals are part of a single subpopulation among other connected subpopulations, this may produce an erroneous bottleneck in demographic analyses. Conversely, if sampling unknowingly includes migrant individuals from other subpopulations, this can create false signals of population expansion in the population of interest (Orozco-terWengel, 2016).

The quality of the DNA used in library preparation has a severe downstream impact on the quality of RADseq data. Whether due to suboptimal field sampling, improper storage or the age of fossil specimens, degraded DNA produces RADseq data with fewer RAD tags per

individual, fewer variable sites and fewer identical RAD tags being retained (Graham et al., 2015). This is predominately the result of large numbers of raw reads being lost as a result of poor quality scores during sequencing (Graham et al., 2015). DNA quality issues can sometimes be addressed with appropriate field sampling technique and specimen preservation, but in the case of ancient DNA or historical samples from museum collections, might be unavoidable (Burrell et al., 2015). Additionally, the PCR amplification step in library preparation can have misincorporation events, where an incorrect nucleotide is inserted into a sequence as a result of hydrolytic damage to the original sequence (Briggs et al., 2009). This leads to incorrect sequences, potential PCR failure (if the primer binding site is damaged), and the labelling of false homozygotes due to PCR drift as one allele is copied more than others at the same locus (Rowe et al., 2011). Biases introduced in the PCR step of library preparation can often be addressed through alteration of the PCR enrichment protocol. Aird et al. (2011) demonstrated that adjustments to the temperature ramp rate, denaturation times and the polymerase used in the enrichment phase drastically reduce the presence of PCR artefacts. PCR duplicates can also be identified via *in silico* quality control using programs such as STACKS (Catchen et al., 2013b), which also allows poor-quality or low coverage reads to be filtered out to eliminate potentially undersequenced or erroneous loci from the dataset.

### *2.2.4 Biogeography of southern Australia*

The Nullarbor Plain, situated in the geographical centre of southern Australia, has been hypothesised to be critical in the formation of the entire region's biogeography from the mid-Miocene (Crisp and Cook, 2007, Byrne et al., 2008). Aridification of Australia intensified during the Pliocene and Pleistocene, leading to the formation of numerous stony and sandy

deserts and the spread of arid and semi-arid habitat over much of inland and southern Australia (Byrne et al., 2008, Fujioka et al., 2005). The south-western and south-eastern regions of mainland Australia are mesic forest zones that possess some of the continent's highest levels of endemism (Byrne et al., 2011), and contain numerous pairs of mesic sister-species. The southwestern region of Australia is considered one of 34 global biodiversity hotspots (Mittermeier et al., 1999), with particularly high levels of avian endemism and plant species diversity. In addition to the Nullarbor, the Eyrean, Swan and Bassian barriers have all been hypothesised to be critical in the formation of contemporary patterns of diversity, by vicariance and dispersal across them (Ford, 1987, Keast, 1961, Schodde and Mason, 1999).

The roles of most biogeographic barriers across southern Australia have a robust amount of support across numerous taxa, including birds (Dolman and Joseph, 2015, Joseph and Omland, 2009), reptiles (Dubey and Shine, 2010), amphibians (Symula et al., 2008) and mammals (Neaves et al., 2009, Cooper, 2003). These species all have identified strong genetic disjunctions between the eastern and western reaches of their distributions, with the Eyrean barrier (some 1000km east of the Nullarbor) attributed to divergence in many bird species in particular. Further south, the timing of the repeated formation and erosion of the isthmus joining Tasmania to the mainland has been inferred to be a significant barrier for dispersal in multiple species (McKinnon et al., 2004, Dawson, 2005). While these and other barriers are frequently invoked as the underlying cause of observed disjunctions in species distributions, further study is needed to cement these putative biogeographic barriers and define their roles in speciation, divergence and community assembly (Glor and Warren, 2011).

## 2.3 Aims & Objectives

In this chapter, my primary aim was to use next-generation sequencing technology to provide estimates of demographic history in populations known to have different degrees of genetic structuring. First, I generated a Restriction site-Associated DNA sequence (RADseq) dataset from the DNA of *Gymnorhina tibicen* specimens. With this genomic dataset, I assessed genetic variation within and among southern populations of *G. tibicen*, representing three of the eight recognized subspecies: *G. t. dorsalis* in the southwestern mainland, *G. t. tyrannica* in the southeastern mainland, and *G. t. hypoleuca* in Tasmania (see Fig. 2.1). I also aimed to infer changes in historical population size over time in each of the sequenced magpie populations.

It was hoped that the results of these analyses would contribute to our understanding of how Australian fauna has responded to environmental changes since the last glacial maximum during the Pleistocene, and whether *G. tibicen* specifically was greatly impacted or more resilient. Previous studies have suggested that southwest and southeast Australia have acted as refugia during the cyclic aridification of the Pleistocene, with signs of expansion observed in mtDNA of Eastern Australian *G. tibicen* populations (Toon et al., 2007). These putative ancestral populations are the focus of this study.

My hypotheses were:

i) That an east-west disjunction among Australian magpie populations is consistent with current arid corridors across southern and central Australia (Nullarbor and Eyrean), as was found in prior genetic studies of the Australian magpie (Baker et al., 2000, Toon et al., 2007).

ii) That as a result of different climate histories, southeastern populations would show less genetic differentiation among them and show signatures of population expansion, while southwestern mainland populations would show greater genetic structure, and a more stable demographic history. The Tasmanian sample sites, representing the lowest latitudes of *G. tibicen*'s distribution, were expected to show signs of very recent expansion, as the birds had not been observed in large numbers in southern Tasmania until the past few decades (Hughes, pers. comm.).

## 2.4 Materials and Methods

### *2.4.1 Sampling Design*

As the study focuses on estimation of historical population size and divergence events (as opposed to the focus on gene flow and genetic structure observed in prior studies of *G. tibicen*), an intermediate sampling design was employed with the intent of striking a balance between the number of sites represented in the sample and the number of individuals per site. As part of previous molecular studies of *G. tibicen* in the same laboratory, a large number of blood samples were collected from sites across Australia. Most sites from prior studies have sample sizes approaching 50 (Toon et al., 2007, Baker et al., 2000, Durrant and Hughes, 2005, Hughes et al., 2001), and were available for use in this study.

For the RAD sequencing component of the project, ten sites were included in the study – four from the southwest mainland, four from the southeast mainland, and two sites from Tasmania (Fig. 2.1). The mainland and Launceston sites were chosen from the collection in the Jane Hughes Molecular Ecology laboratory at Griffith University, and field sampling was performed in Hobart (where magpies have been recorded in numbers only in the last 20 years) to add a second Tasmanian site to the dataset. A total of 95 individuals were prepared for genome sequencing.

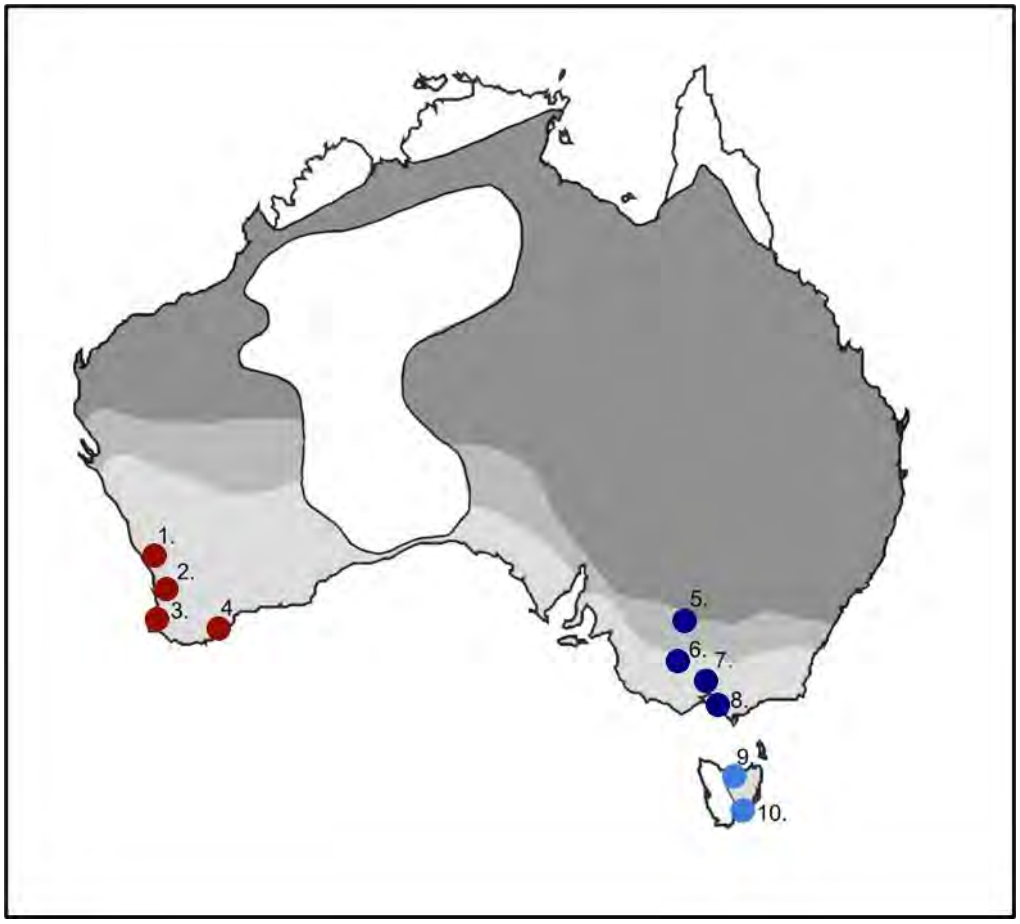


Figure 2.1 Distribution of plumage morphs of the Australian Magpie (*Gymnorhina tibicen*), with numbered sample sites. Regions with black-backed forms are highlighted dark grey, white-backed forms in light grey, and intermediate zones in medium grey. Sample sites are numbered as follows: 1. Perth (Ph), 2. Mandurah (Mh), 3. Busselton (Bn), 4. Albany (Al), 5. Ouyen (Ou), 6. Horsham (Hr), 7. Rowsley (Ro), 8. Philip Island (PI), 9. Launceston (Ln), 10. Hobart (Hb). Refer to Appendix 1 for detailed information about sites. Map adapted from Toon and Hughes (2008).

### *2.4.2 Ethical Approval & Scientific Permits*

Samples were collected from the Hobart region under a Permit to Take Wildlife for Scientific Purposes #FA17081, issued by the Tasmanian Department of Primary Industries, Parks, Water and Environment (DPIPWE). Ethical Approval for field sampling was granted by both the Griffith University Animal Ethics Committee (Protocol number ENV/02/17/AEC) and the DPIPWE Animal Ethics Committee (AEC Project Number 8/2016-17) in accordance with the *Australian Code for the Care and Use of Animals for Scientific Purposes, 8th edition, 2013*.

### *2.4.3 Field sampling protocol*

The magpies sampled as part of this study were caught using a caged live decoy magpie within an outer trap constructed of chicken wire (fig. 2.2). The decoy was captured using a wider variant of the trap used in sampling, with the assistance of a 'dummy' magpie constructed of a plastic bottle and black and white paper (fig. 2.3). The live decoy used in the study was a subdominant male (determined visually from relative body size and behaviour, allowing the larger dominant male to enter the trap first), obtained from a territory with more than one adult male to reduce the likelihood of disrupting the territory through its absence. The trap and decoy were set up at each site in order to prompt a defensive territorial response, and bait food was scattered within the outer trap to encourage individuals to remain within the cage once lured. A funnel-shaped section of wire was used to allow wild magpies to enter the trap and prevent their escape once they had entered. In some cases, a recording of wild magpie calls was also used to assist in luring wild individuals towards the cage.

Captured birds were removed from the trap by hand and blood samples were collected via the clipping of an outer toenail on the foot of the bird. The blood samples were stored in 1.5mL screw-cap tubes containing 1mL of lysis buffer (0.1M Tris-Hcl ph 8.0, 0.1M EDTA, 0.5% SDS, 0.01M NaCl). The clipped toenail was then swabbed with disinfectant before releasing the bird back into the territory. In the lab, blood samples were stored at -80°C for later DNA extraction. Samples that were collected as part of earlier studies were primarily stored in the same lysis buffer solution, though some early samples were not so well preserved, with little to no lysis buffer and considerable congealing.

Figure 2.2 Example of trap used in field sampling, with live ‘decoy’ individual in place.

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Figure 2.3 ‘Dummy’ bird used to attract magpies for the purposes of procuring a live decoy.

*2.4.4 DNA extraction*

A protocol for extracting DNA of sufficient quality and concentration for RAD sequencing was developed as part of a past Honours project in the same laboratory (Prince, 2015), based on a framework provided by earlier studies (Etter et al., 2011, Catchen et al., 2013a). The aim was to achieve high concentrations of high-quality DNA to mitigate the loss of information through size-selection, and to ensure a high level of consistency for comparisons across multiple samples.

Before proceeding, any blood samples that were not stored in buffer were suspended in a solution of Blood Lysis Buffer, in a rough 2:1 ratio of buffer to blood. 100-150µl of each buffered sample was added to a 1.5mL Eppendorf tube. Phosphate-buffered Saline (PBS) solution was then added to each sample to a total volume of 500µl, and the samples were then bead-beaten with a Tissue Lyser II (QIAGEN), using one 3mm Tungsten Carbide bead (QIAGEN) per sample, at 30 beats/second for 10 seconds. This mechanical disruption aids in the breaking down of red blood cells and increases the final concentration of extracted DNA at the expense of overall quality. In addition to breaking up the cells of the sample and regulating the stability of the extraction environment, suspension in Lysis Buffer also renders the sample more robust to the loss of quality incurred by bead-beating. Wherever possible, clotted blood (increased coagulation being a side effect of suspension in Lysis Buffer) was drawn from the available samples, as clots produce higher yields of DNA than unclotted samples.

The bead-beaten samples were transferred to new 1.5mL Eppendorf tubes, and samples were digested for a minimum of 8 hours at 55°C in a heat block until all coagulated blood was

broken down. After the digest was complete, 4µl of RNase A was added to each sample and left for 15 minutes to remove any RNA that could interfere with downstream PCR. 200µl of 2M Guanidine Hydrochloride, 200µl of 100% Ethanol and 100µl of Precipitation Buffer (4M Ammonium Acetate) was then added to each sample, before resting for an additional 15 minutes at room temperature. Each sample was then transferred to an EconoSpin column (Qiagen) and centrifuged at 16,000g for 15 minutes. The resulting flow-through solution was discarded from the collection tube, and 500µl of Binding Buffer (2M GuHcl, 75% Ethanol) was added to each column. The samples were centrifuged again (16,000rcf, 15 minutes), discarding the flow-through solution, before 500µl of Wash Buffer (80% Ethanol, 1M Tris) was added to each tube and centrifuged at the same settings, again discarding the flow-through. The Wash Buffer step was repeated a second time, and the Spin Column was moved to a 1.5mL Eppendorf tube. 80µl of Elution buffer was added directly to the membrane of each column and allowed to soak for 30-60 minutes before being centrifuged at 6,000rcf for 1 minute.

The eluted DNA samples were visually assessed for quality and concentration through Agarose Gel Electrophoresis. 2-3µl of sample was mixed with 1µl of Gel Loading Dye and run on a 1.2% Agarose gel at 80V for 1 hour. Once the quality of DNA was confirmed, the samples were subsequently assessed for DNA concentration using a Qubit Fluorometer. Samples with a visibly high quality (appearing as a tight, bright band >10kb in size on the Agarose gel) and a concentration of greater than 20ng/µl were retained for use in the RAD library preparation phase.

*2.4.5 RAD library preparation and sequencing*

Once DNA of sufficient quality and concentration was extracted from each of the 95 samples, they were sent to the Australian Genomic Research Foundation (AGRF) for library preparation and sequencing. In brief, the AGRF uses a double digest RAD (ddRAD) based library preparation protocol (Peterson et al., 2012), in which two restriction enzymes are used to digest the DNA (in this case, *ecoRI* and *HpyCH4IV*). Barcoded adapters are ligated to each fragment to differentiate between samples, and the pooled fragments are size-selected using a Blue Pippin electrophoresis machine. The library is then amplified via PCR using indexed primers and sequenced on the Illumina NextSeq platform. Over all samples sequenced, >91% of bases had a quality score above Q30, or 99.9% accuracy.

*2.4.6 RAD data preparation*

The sequenced RAD library produced 55Gb of raw sequence data from 95 *Gymnorhina tibicen* samples, in the form of 360 million 150bp single-end reads. The short-read sequences obtained from the Illumina platform were cleaned and demultiplexed before further analysis. The bioinformatics software STACKS (Catchen et al., 2013b) is a versatile and widely used pipeline designed to assemble loci from reads such as those produced by our restriction enzyme digest, and was used in the preparation of the RAD data.

First, the raw sequence data from the pooled samples was sorted to recover individual samples based on their barcodes, using the *process_radtags* function. Reads with missing restriction enzyme cut sites were discarded, as were any low-quality reads based on their Phred score. Phred scores are derived from the probability that a base call is incorrect, and are widely used to assess the quality of sequences and identify questionable reads for removal

(Richterich, 1998). An average of 3,791,786 reads per sequenced individual was recovered after demultiplexing. Two individuals from the Albany and Perth populations had an order of magnitude fewer reads (<300,000) and were omitted from the dataset for downstream filtering and analyses.

Once the data has been cleaned and demultiplexed, STACKS offers two approaches for assembling individual reads into loci. If a reference genome is available, STACKS can use it to build loci by aligning them to the reference. However, such reference genomes are not presently available for *Gymnorhina tibicen* or closely related species. With that in mind, a *de novo* approach was used for the next steps in the STACKS pipeline. Using this *de novo* approach, the assembly algorithm conservatively assembles putative alleles, and then assembles alleles into putative loci. If proper filtering is used when creating the catalogue of loci, a reliable set of loci can be procured (Rochette and Catchen, 2017).

The appropriate parameters for use in the STACKS pipeline vary when working on different non-model study species (Paris et al., 2017). To test an array of parameters in greater depth, the individual steps in the pipeline (*ustacks*, *cstacks*, *sstacks*, and *rxstacks*) were run by hand rather than using a wrapper script. Appropriate selection of parameters should prevent loci being created in only one or two individuals or populations. The *de novo* approach comes with some caveats, as each sample will contribute some false positive loci to the catalogue (Mastretta-Yanes et al., 2015). Additionally, without a reference genome, the position of loci on the genome cannot be determined, and so each locus must be examined individually. Throughout the STACKS pipeline, values must be set for several key parameters, which have a significant impact on the locus-building process.

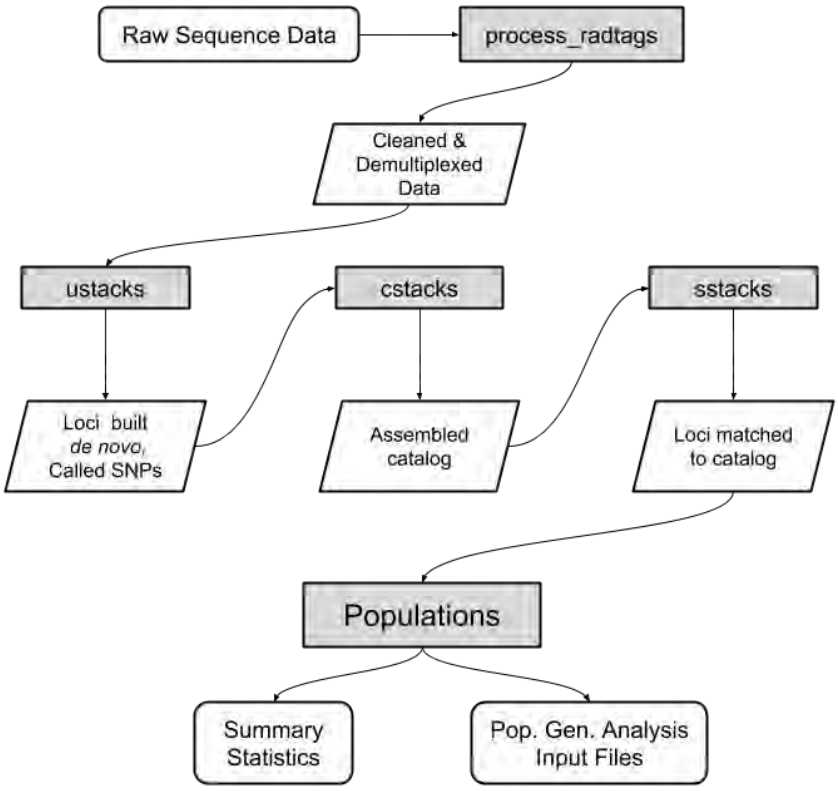


Figure 2.4 Flow diagram of the STACKS pipeline. Modules within the STACKS software package are shaded in grey, while the input/outputs of each stage are in white. (Catchen et al., 2013b)

*2.4.7 Parameter selection in Stacks*

Stack assembly using a *de novo* approach is controlled by several parameters that dictate how the program filters and collates sequence data. There are several factors that influence which values are 'ideal' for each parameter, including the level of polymorphism in the RADseq dataset, the hypotheses being tested, the number of individuals that have been sequenced and the restriction enzyme used, coverage levels, sequencing platforms and more. As each dataset is unique, the parameters used to filter them must be set carefully, and there is no easy way to determine the 'best' values for each. A procedure first published in Rochette and Catchen (2017) was followed when running the Stacks program, to ensure each step was executed by hand correctly and to compare the results of the pipeline when using different parameter values. Paris et al. (2017) established a decision framework for testing different values in STACKS, and this was used to select appropriate values for each parameter during catalogue creation from the *Gymnorhina tibicen* data.

In the *de novo* pipeline, the most critical parameters are those which determine how similar sequences are permitted to be assembled into the same locus. These mismatch parameters are what ultimately decides how loci are created, and which reads are assigned to loci and which are discarded. Mismatch tolerance must be relaxed enough to account for genetic variation and sequencing errors, but still strict enough that paralogous loci can be separated from one another.

First, the unit *ustacks* was used to assemble loci. The two main parameters at this stage were *m*, which controls the number of raw reads required to form an initial 'stack' (a putative allele), and *M*, which controls the number of mismatches allowed between stacks to merge

them as alleles of a heterozygous sample. After these parameters have been set, a second unit named *cstacks* was used to match homologous loci from each sample, merging them into a common catalogue. To account for fixed differences in loci between individuals, the parameter *n* controls the number of mismatches allowed between any two alleles of the population.

Coverage (the average number of times a base has been sequenced) is an important metric to consider when assembling loci, as studies have demonstrated that both lower coverage, and to a lesser extent sequence quality, lead to higher rates of genotyping error (Fountain et al., 2016). Genotyping errors are considerably more likely when coverage is poor (5 to 10x), and sequencing error is less of a concern when coverage is >10x. Datasets with lower mean coverage can still be used (Xu et al., 2014, Razkin et al., 2016), and may be the only option due to budget constraints or poor sample quality, but uncharacteristically low coverage samples were not retained for this study. The mean sequencing coverage for the 95 *Gymnorhina tibicen* samples ranged from 8x to 49.9x (fig. 2.5). Once the two individuals with significantly lower numbers of raw reads were omitted from the dataset, the minimum coverage increased to 23x. These values were not low enough that genotyping errors were a major concern in subsequent steps of the STACKS pipeline. The impact of coverage on the *de novo* assembly process is greatest through the *m* parameter, as if *m* is set to 1, each raw read is a putative allele of its own without secondary reads. As *m* increases, putative alleles require more evidence to identify, and secondary reads are assigned more frequently, and therefore alleles with lower coverage will be passed over. While high values for *m* are appropriate for some studies where loss of within-species polymorphism are not a concern (Wagner et al., 2013) or exogenous contamination must be avoided (Trucchi et al., 2016), I aimed to set *m* only high enough that errant reads would not be considered putative alleles, in

order to avoid unnecessary loss of alleles. The minimum stack depth (*m*) was set to 3 by default, and this appears to be the optimal value for a wide range of single-species datasets (Paris et al., 2017), so this value was retained for further analyses.

Higher values of *M* than the default set by Stacks may be needed if the study species has higher levels of polymorphism. Too low, and alleles will not be collated into loci within individuals, instead creating homozygous loci and excluding the alternative alleles. If too many mismatches are tolerated between stacks, repetitive and/or paralogous loci can be merged by mistake. Paris et al. (2017) demonstrates that different values of *M* are optimal for different species datasets, and a range of values should be tested for this parameter. The number of mismatches tolerated in the catalog (*n*) should also be tested using iterative values for the parameter. If populations are closely related, a suitable baseline would be $n = M$, as they control the matching of alleles across individuals and within individuals. If fixed differences are less common, as with larger population sizes, *n* should be set to a value less than *M*. Conversely, if the individuals included in the study are highly divergent, a higher *n* may be needed to detect polymorphisms. In my optimization runs, a subset of 12 samples were tested through *ustacks* and *cstacks*, with the distance allowed between stacks (*M*) set from 1 to 9, and the distance allowed between catalog loci (*n*) set from 1 to 5.

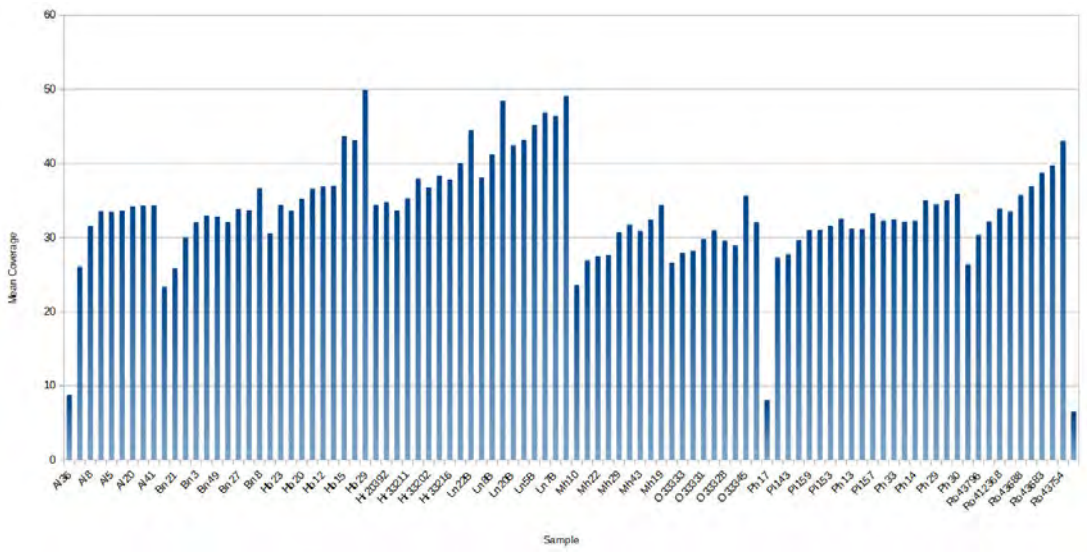


Figure 2.5 Mean coverage per sample across the *G. tibicen* dataset. Low coverage (<10x) samples are from Albany (Al36), Perth (Ph17), and a negative control, in order.

Table 2.1 STACKS parameters used during de novo locus construction and filtering.

*2.1a Parameters controlling de novo formation of stacks and loci*

| Parameter | Default Value | Value Used | STACKS component | Description |
|---|---|---|---|---|
| *m* | 3 | 3 | ustacks | Minimum number of raw reads required to form a putative allele |
| *M* | 2 | 4 | ustacks | Mismatches allowed between stacks (putative alleles) to merge into putative locus |
| *n* | 1 | 4 | cstacks | Mismatches allowed between putative loci during catalogue construction |

*2.1b Parameters filtering loci used for population-level analyses*

| Parameter | Default Value | Value Used | STACKS component | Description |
|---|---|---|---|---|
| *r* | 0 | 0.8 | populations | Percentage of individuals that must possess a locus for it to be included in calculation of population-level statistics |
| *max_obs_het* | 1 | 0.70 | populations | For a locus, the maximum number of heterozygous individuals that may be present |
| *min_maf* | 0 | 0.05 | populations | For a locus, alleles occuring below this frequency are discarded |

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Table 2.2 Total and new r80 loci for each iteration of M.

| M | total r80 loci | new r80 loci | total poly r80 | new poly r80 |
|---|---|---|---|---|
| M2 | 36077 | 36077 | 30391 | 30391 |
| M3 | 36864 | 787 | 31288 | 897 |
| M4 | 37040 | 176 | 31535 | 247 |
| M5 | 37048 | 8 | 31589 | 54 |
| M6 | 37064 | 16 | 31643 | 54 |
| M7 | 37028 | -36 | 31656 | 13 |
| M8 | 37004 | -24 | 31686 | 30 |

After running *ustacks* and *cstacks* on the subset of samples using increments of parameters *M* and *n*, I compared the results obtained with each combination. First, the number of polymorphic *r80* loci (shared across >80% of samples) and the distribution of the number of single nucleotide polymorphisms (SNPs) were plotted, both of which are useful for choosing a suitable parameter set. As seen in Figure 2.6, the number of widely shared loci plateaus around $M = 4$, and at $M$ & $n = 4$, the proportions of loci with 1-6 SNPs begin to stabilise. The total number of *r80* loci and new loci added to the catalog as *M* increases can be seen in Figure 2.7. The number of new loci begins to plateau after $M = 4$, and while the total *r80* count decreases as *M* approaches 8, the number of 'new' loci added increases. As mentioned earlier, this is due to homozygous loci being folded together as the algorithm becomes more tolerant of mismatches between putative alleles, combining them into an erroneously polymorphic locus.

Once appropriate values of *M*, *m* and *n* were selected, the *populations* subunit was used to further filter the alleles into an optimised dataset. This was done following the method described in Paris et al. (2017). The parameter *min_maf*, controlling the minimum minor allele frequency, was set to 0.05 to filter out alleles that only occurred at very low frequency, and *max_obs_het,* the maximum observed heterozygosity at a locus, was set to 0.70 to filter out repetitive or paralogous loci that may have been merged together (if erroneous, then they were likely to appear as heterozygous in a large proportion of individuals).

### *2.4.8 Exploratory data analysis*

Summary statistics, including the number of polymorphic sites, observed and expected heterozygosity, nucleotide diversity, inbreeding ($F_{IS}$), and between-population $F_{ST}$ based on

SNPs were generated as part of the *populations* subunit of Stacks. A hierarchical analysis of molecular variance (AMoVa) was also conducted on all populations using two levels of grouping, with the different geographic regions as the highest level (western mainland, eastern mainland, and Tasmania), and sample sites within regions (western mainland = Al, Bn, Mh, Ph, eastern mainland = Hr, Ou, Ro, PI, Tasmania = Hb, Ln) as the second level.

Further analyses were conducted using R and Rstudio (Team, 2020). Additional summary statistics and basic measures of population differentiation were generated using the R packages *adegenet* (Ahmed and Jombart, 2011) and *pegas* (Paradis, 2010).

The R Bioconductor package LEA ver 2.4.0 (Frichot and François, 2015) was used to investigate population structure further. A set of 100 runs was used to evaluate the most likely number of ancestral populations (*K*) from 1 to 10, with ten replicate runs of each value of *K*. The resulting cross-entropy criterion was calculated and compared between runs, with a smaller value for cross-entropy indicating a better prediction.

### *2.4.9 Demographic history*

To infer historical population size through time, I ran an Extended Bayesian Skyline Analysis in Beast2, version 2.5.2 (Bouckaert et al., 2014). The EBS model is widely used for inferring population history from multiple loci, though utilising RADseq data is a relatively recent development (Trucchi et al., 2014). The possibility that individuals appear homozygous at a locus but are in fact undersequenced heterozygotes must be considered, as this would introduce bias into the coalescent reconstruction. As the mean coverage of my dataset was

high (23x), this was not a great concern, so I elected to retain both alleles for each individual at each locus, rather than downsample to one haplotype per individual.

As the number of loci used drastically increases computational time, I selected a subset of more informative loci, as more variable loci will contain more information about coalescent events. This practice is common to many types of marker, such as the use of the more variable mitochondrial *COI* region over *Cyt b*. RAD loci with at least 4 SNPs, to a maximum of 6 SNPs per locus, were selected across the individuals in the populations analysed. These loci were converted to separate '.fasta' alignment files, with each individual represented by two alleles, and loaded into a BEAUti template. Site models were linked for all loci with the same number of SNPs, and the overall clock model was linked for all loci, as neutral evolution of the RADome is assumed and the overall rate does not differ between loci. A HKY+G site model was used for all classes, and the MCMC length was set to 500,000,000 iterations, with a burnin of 50,000. To estimate the 'real' timing of demographic changes, a RAD locus-specific clock rate was calculated using methods similar to Lado et al. (2020). I calculated the average number of differences between eastern and western *G. tibicen* sequences, divided by the number of RAD loci, then took the average among the SNP classes (4, 5 and 6 SNPs per locus), divided by an east-west divergence date of 36,533yrs (inferred from mitochondrial DNA in an earlier study (Toon et al., 2007)), and a magpie generation time of 4 years (Hughes, pers. comm.).

All Beast2 runs were performed on the High Performance Computing Facility at Griffith University. The resulting log files were inspected using Tracer (Rambaut et al., 2018) to evaluate support values for each parameter before plotting the output.

Initial EBSP runs produced mixed results. Runs that included 8 or more individuals from multiple sample sites in a region consistently produced erratic demographic signals indicative of a sharp decline approaching the present, with a brief upward spike in effective population size just before the estimates reach the present. To address this, I repeated the EBSP analyses with reduced sample sizes of 2 individuals per population and three subsets of 50 loci with between 4 and 6 SNPs.

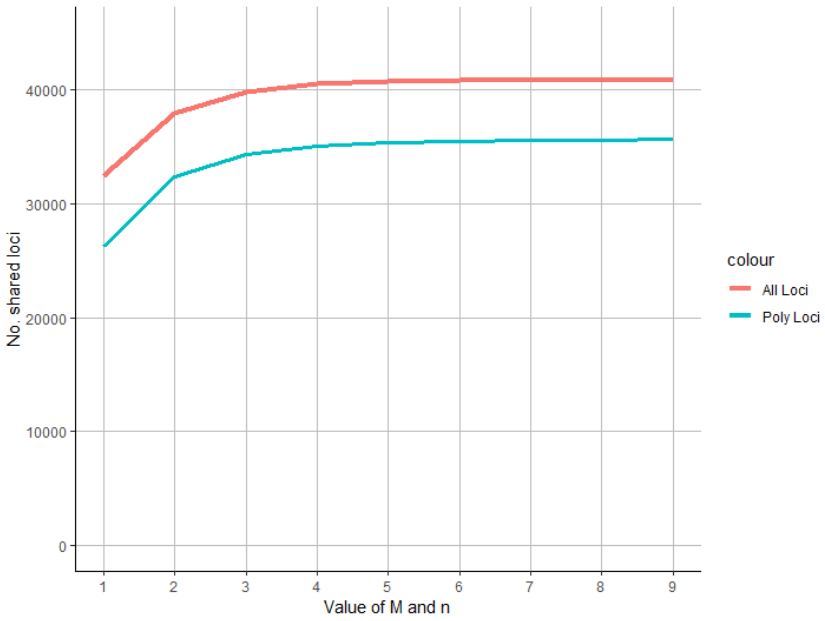
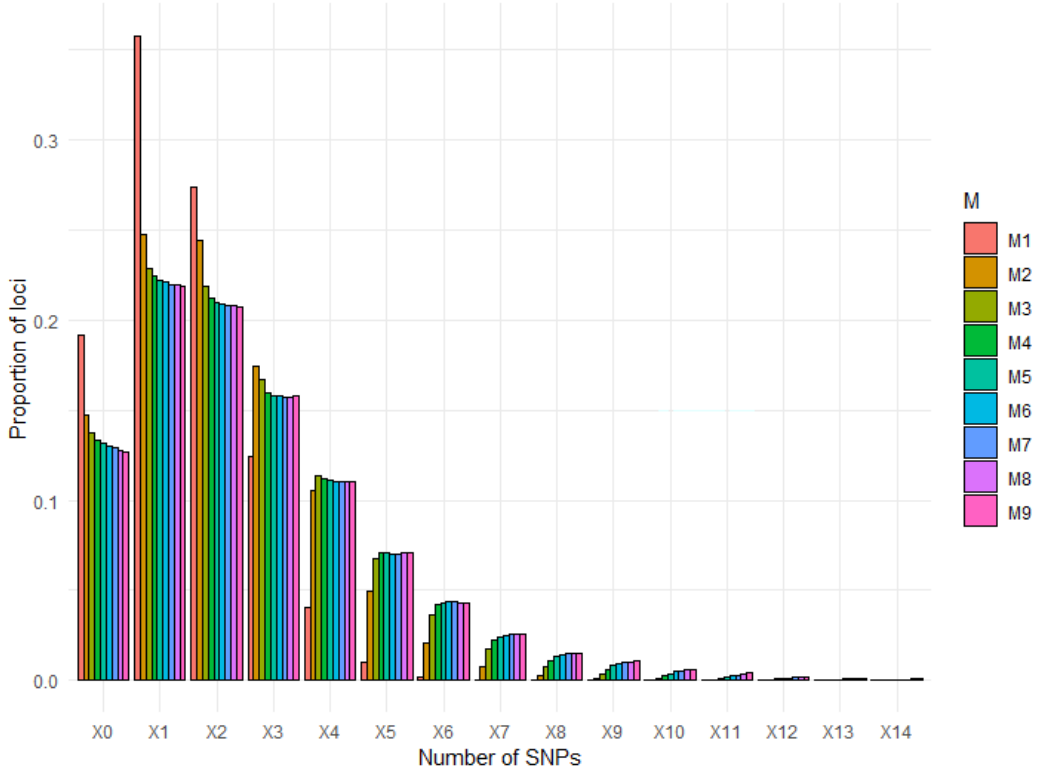



Figure 2.6 Total number of loci (red) and number of polymorphic loci (blue) shared by >80% of G. tibicen samples for each iteration of $n = M$ from 1 to 9




1135 Figure 2.7 Distribution of the number of SNPs per locus for each iteration of *M*, from 1 to 9. When *M* is left at 1, the number of homozygous loci (0 SNPs) detected greatly increases, as alternate alleles are erroneously classed as new loci. At higher values of *M (*8-9*)*, few loci with up to 19 SNPs were detected, but these were not visible on the scale of this graph and were omitted.

## 2.5 Results

### *2.5.1 Data preparation & summary statistics*

After running STACKS with the chosen parameters (see **Table 3.1**) using the *r80* method, a genomic dataset was produced containing 2,178,832bp of information, incorporating 29,406 SNPs across 15,345 loci. The distribution of SNPs across loci can be seen in **Figure 2.8.** The 1 SNP class was the most common, with 6,287 loci.

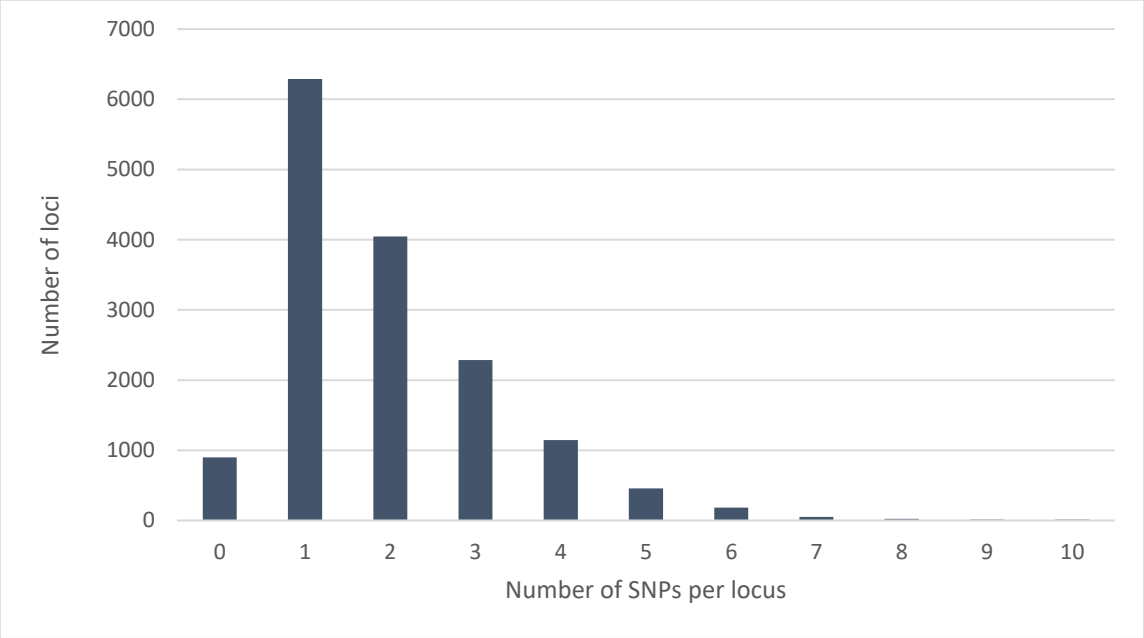



Figure 2.8 Distribution of SNPs across loci in the *Gymnorhina tibicen* dataset.

When grouped by region, western mainland populations contained more private alleles than the eastern or Tasmanian populations (Table 2.3), but when each site was treated as a separate population, Hobart and Launceston had the most private alleles. The degree of polymorphism in the dataset was greatest in the eastern populations (Horsham, Ouyen, Rowsley and Phillip Island), and lowest in the Tasmanian populations.

Table 2.3 Summary statistics for each population. Statistics include number of private alleles, no. polymorphic sites, percentage of loci that are polymorphic, mean number of individuals per locus, mean observed and expected heterozygosity, nucleotide diversity and mean measure of F*is*.

| Region | Private Alleles | Population | Private Alleles | Polymorphic Sites | % Polymorphic Loci | No. Indiv | Obs. Het | Exp. Het | Pi | *Fis* |
|---|---|---|---|---|---|---|---|---|---|---|
| West | 2673 | Albany | 0 | 19898 | 0.9132 | 7.834 | 0.0025 | 0.0028 | 0.003 | 0.0013 |
| | | Busselton | 2 | 19341 | 0.8877 | 9.8098 | 0.0025 | 0.0028 | 0.0029 | 0.0011 |
| | | Mandurah | 0 | 20064 | 0.9209 | 8.859 | 0.0027 | 0.0028 | 0.003 | 0.0009 |
| | | Perth | 0 | 19403 | 0.8905 | 7.87 | 0.0026 | 0.0028 | 0.003 | 0.0009 |
| East | 815 | Horsham | 0 | 22443 | 1.03 | 8.7696 | 0.0027 | 0.0029 | 0.0031 | 0.0012 |
| | | Ouyen | 0 | 22366 | 1.0265 | 8.7455 | 0.0026 | 0.0029 | 0.0031 | 0.0013 |
| | | Phillip Island | 1 | 20858 | 0.9573 | 9.7343 | 0.0026 | 0.0028 | 0.003 | 0.0011 |
| | | Rowsley | 0 | 22422 | 1.0291 | 9.6433 | 0.0026 | 0.0029 | 0.0031 | 0.0015 |
| Tasmania | 636 | Hobart | 6 | 18384 | 0.8438 | 9.8204 | 0.0024 | 0.0027 | 0.0029 | 0.0012 |
| | | Launceston | 5 | 19475 | 0.8938 | 9.7767 | 0.0026 | 0.0028 | 0.003 | 0.0009 |

Table 2.4 Pairwise $F_{ST}$ values for all pairs of populations of *Gymnorhina tibicen*. Green cells are closely related and red cells least related.

| | Al | Bn | Mh | Ph | Hr | Ou | Pl | Ro | Hb | Ln |
|---|---|---|---|---|---|---|---|---|---|---|
| Al | | 0.029559 | 0.023137 | 0.0258 | 0.034336 | 0.030668 | 0.046862 | 0.037377 | 0.084626 | 0.074926 |
| Bn | | | 0.023553 | 0.030809 | 0.05255 | 0.049412 | 0.067499 | 0.056253 | 0.105431 | 0.095134 |
| Mh | | | | 0.02071 | 0.042429 | 0.039238 | 0.059459 | 0.047966 | 0.096998 | 0.086492 |
| Ph | | | | | 0.041954 | 0.04021 | 0.057117 | 0.044923 | 0.093697 | 0.08487 |
| Hr | | | | | | 0.00654 | 0.016691 | 0.007993 | 0.050932 | 0.041535 |
| Ou | | | | | | | 0.016755 | 0.009309 | 0.051958 | 0.042644 |
| Pl | | | | | | | | 0.016534 | 0.063669 | 0.053287 |
| Ro | | | | | | | | | 0.051052 | 0.041345 |
| Hb | | | | | | | | | | 0.024409 |
| Ln | | | | | | | | | | |

*2.5.2 Genetic structure & diversity*

Figure 2.9 shows the results of a principle component analysis performed using the R package adegenet ver. 2.1.1 (Ahmed and Jombart, 2011, Jombart, 2008). 93 individuals of *Gymnorhina tibicen* are included in the analysis from all 10 sample sites. The overall explanatory power of the first two principal components was small, with PC1 explaining only 9.25% of the variation in the dataset and PC2 explaining 4.27%. The third and fourth principal components accounted for 2% each, and past the sixth principal component, none accounted for >1% of the variation. It took 77 principal components to cumulatively account for 90% of the variation in the dataset.

The sequenced individuals differentiate from one another based on sample site and sampling region (eastern mainland, western mainland and Tasmania). Tasmanian samples appear more differentiated from the mainland than the two mainland regions are from each other. Other phenomena of interest include Phillip Island (shown in purple), which was more differentiated from the other eastern populations, despite the island being separated from the mainland by only <1km of water. Individuals sampled from Hobart (shown in pink) were also more differentiated than other populations.

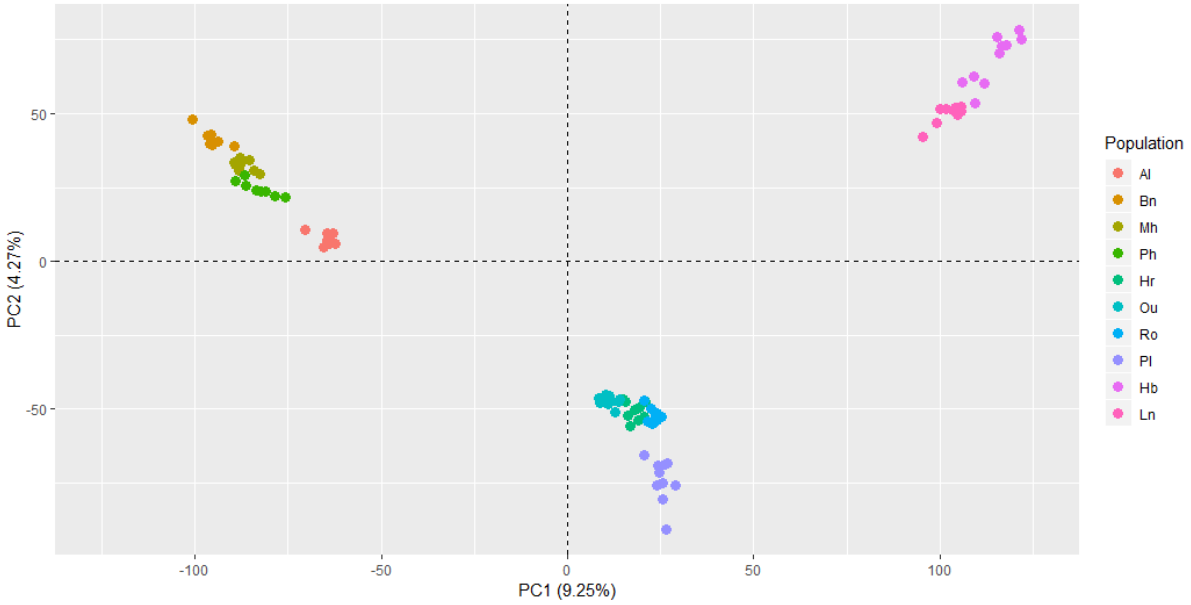



Figure 2.9 Principle Component Analysis (PCA) of the RADseq data, totalling 29,406 SNPs. PC1 accounts for 9.25% and PC2 for 4.27% of the variation in the dataset, for a combined total of 13.2% of the total variation.

The results of the analyses performed in LEA are shown in figures 2.10 & 2.11. Figure 2.8 shows the mean cross-entropy for each *K* value, with the analysis indicating that *K*=3 was the most likely. The admixture coefficient matrix for *K*=3 is visible in Figure 2.9. Similar to the principle component analysis (see Fig. 2.7), genetic structure was evident among the eastern, western and Tasmanian regions, with each region predominant in its own cluster. In the western mainland sites, Albany showed the greatest level of coancestry with the eastern grouping, followed by Perth. Similarly, Phillip Island showed a higher level of coancestry with the western populations than the other eastern mainland populations.

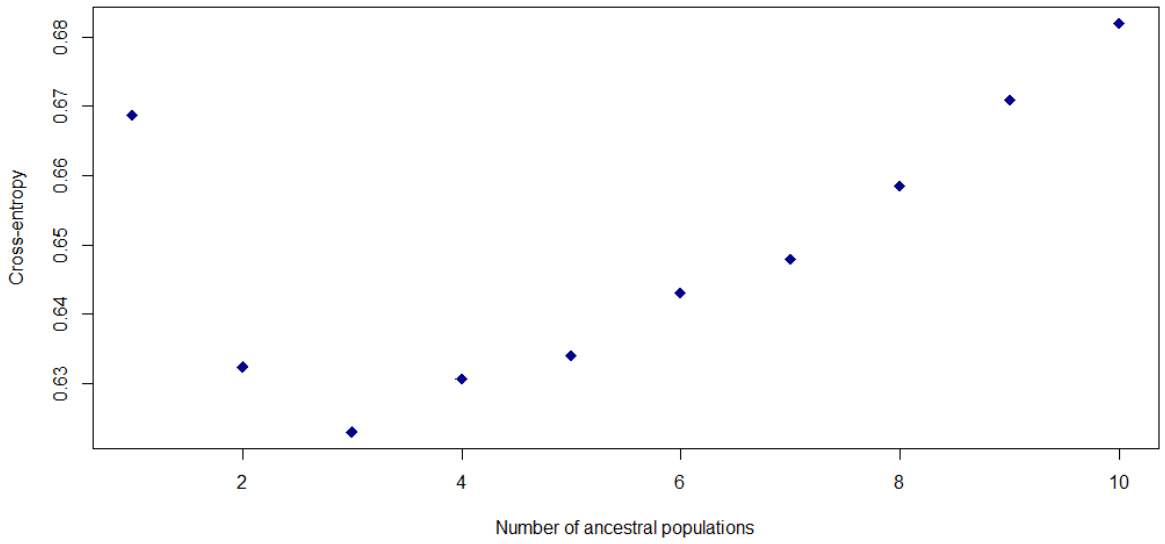
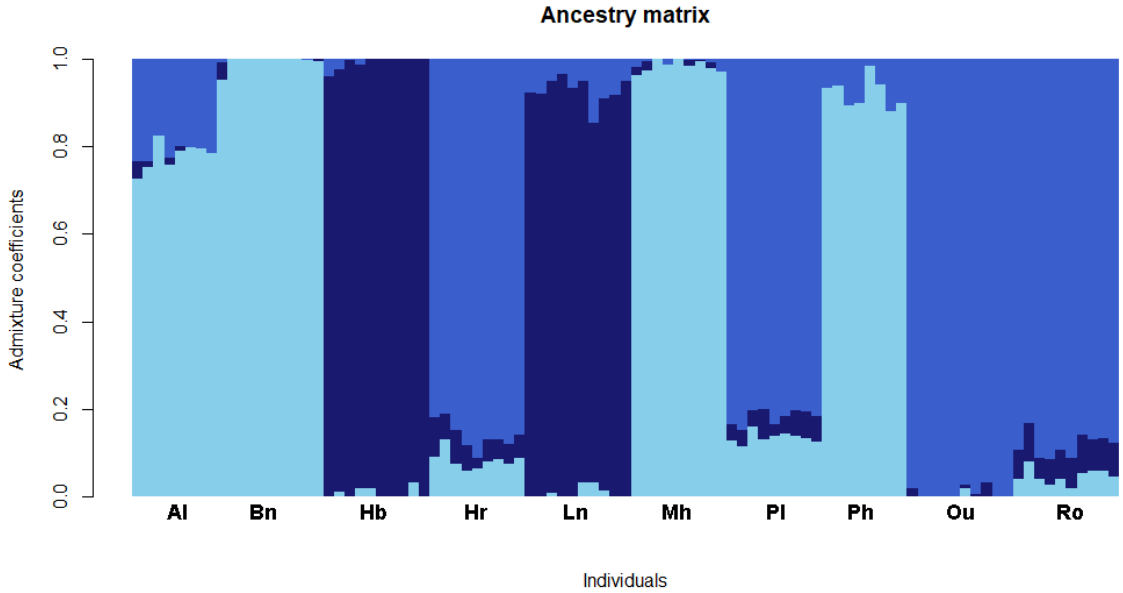



Figure 2.10 Mean cross-entropy criterion for each possible number of ancestral populations in the *G. tibicen* dataset. Values of *K* from 1 to 10 were run 10 times each to find the most appropriate value, with *K* = 3 having the most support.




1195

Figure 2.11 Coancestry matrix of *Gymnorhina* tibicen specimens, *k* = 3. Generated with LEA, derived from 29,604 SNPs. Identified genetic clusters correlate strongly with western (light blue), Tasmanian (dark blue) and eastern (intermediate) sites.

To supplement the analyses of population structure further, the dataset was used in conjunction with the software package FineRADStructure (Malinsky et al., 2018) to generate a coancestry matrix comparing every individual. This produced the same broad patterns as in other analyses (Figure 2.12), with the Tasmanian region exhibiting the greatest levels of interrelatedness.

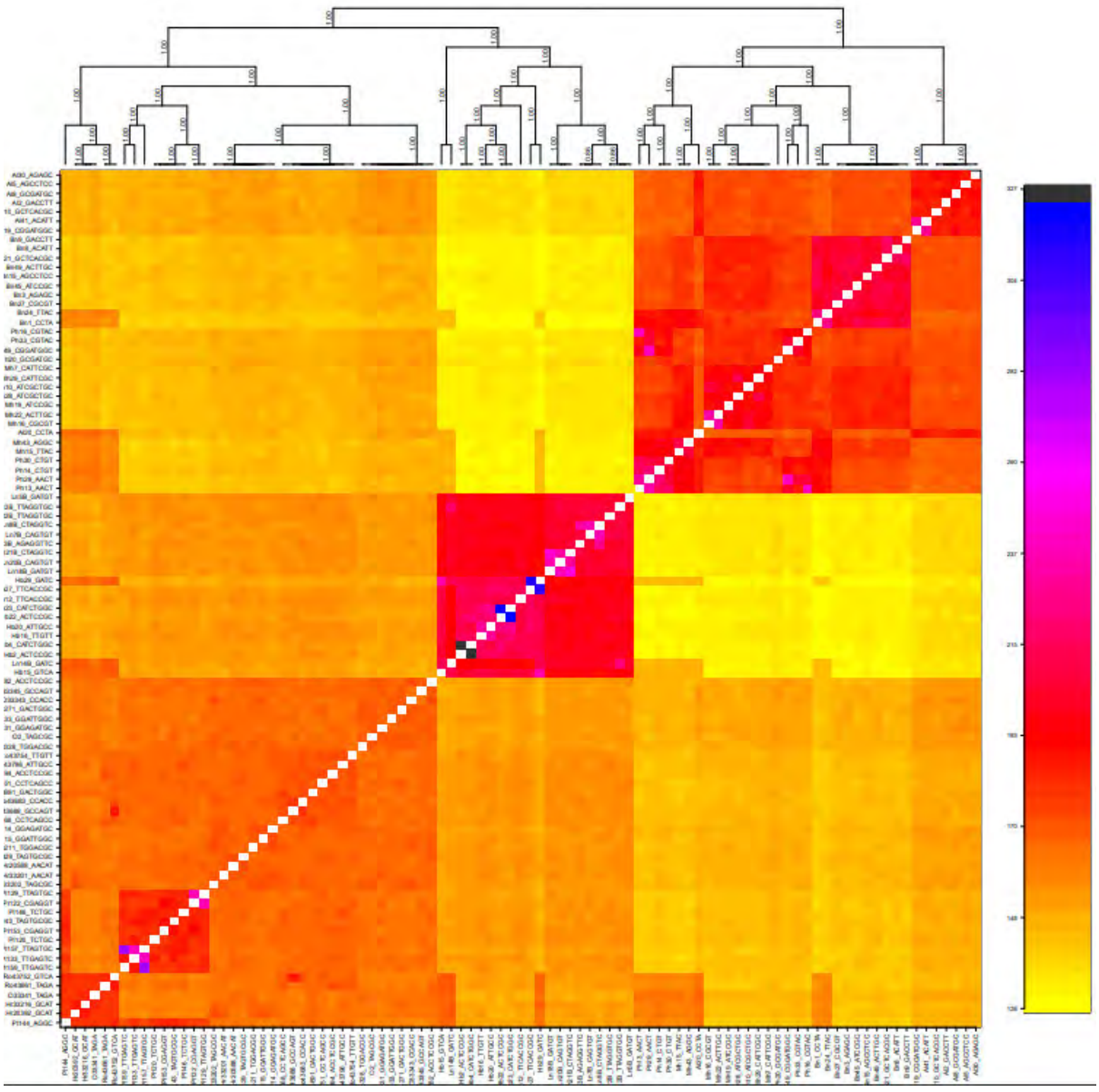
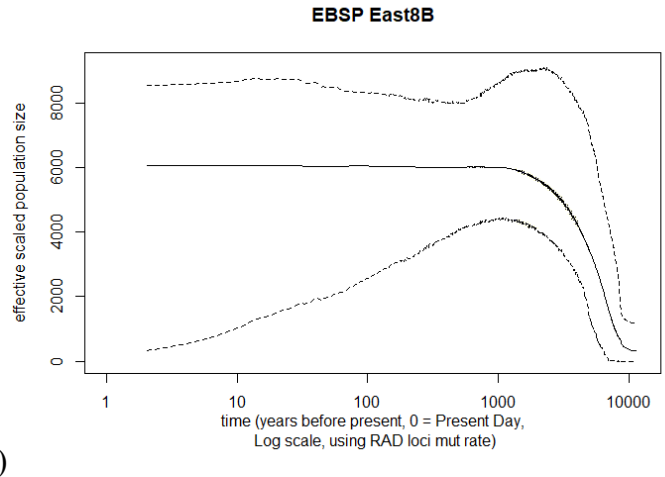


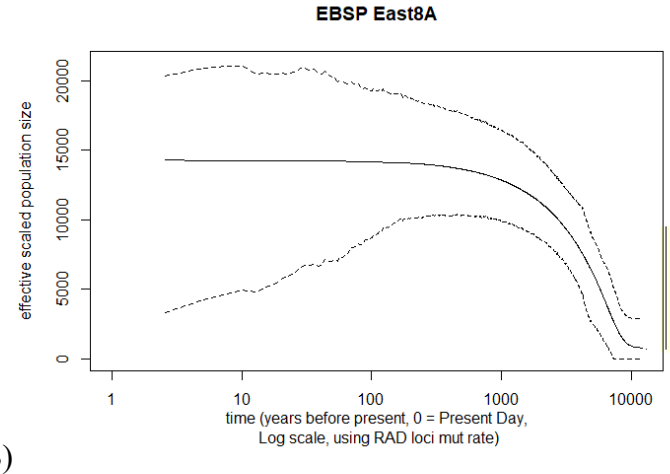
Figure 2.12 FineRADStructure coancestry matrix comparing individual relatedness of *Gymnorhina tibicen* specimens. Based on 29,406 single nucleotide polymorphisms (SNPs). Heatmap colours indicate degree of relatedness, with major related groups visible: western (top right), Tasmanian (centre), eastern (bottom left**).**

### *2.5.3 Demographic history*

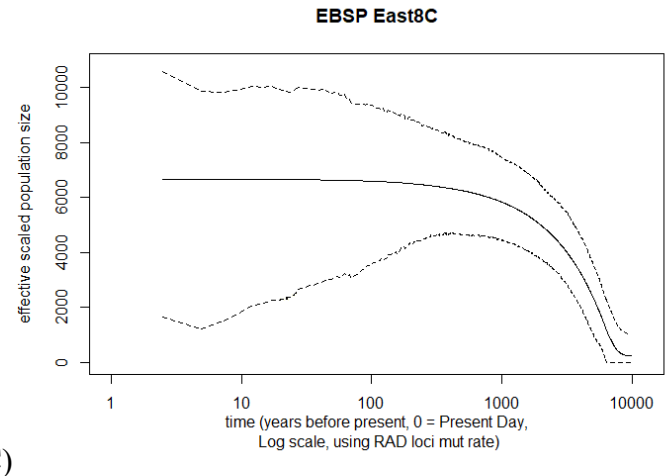
Extended Bayesian Skyline outputs for the eastern populations converged on the same demographic signature of a continuous increase in population size approaching the present (Figure 2.13). Conversely, both the western and Tasmanian clusters showed signs of a historical increase in population size followed by a sharp decrease, before stabilising towards the present (Figures 2.14 & 2.15). Effective population size in the western region appears to have peaked between 1200-1900 years before present, while the Tasmanian population peaked around 2000-2300ybp. Support values were high (ESS > 200) in each of the three runs performed for each cluster, except for one Tasmanian run which failed to converge on a well-supported demography and was subsequently excluded from the plots. While the direction and timing of demographic change was consistent across subsets of loci, the scale of the estimated effective population size for each subset differed, particularly in the case of the eastern group, where estimates of population size approaching the present ranged from approximately 6000 to 15000.




A)

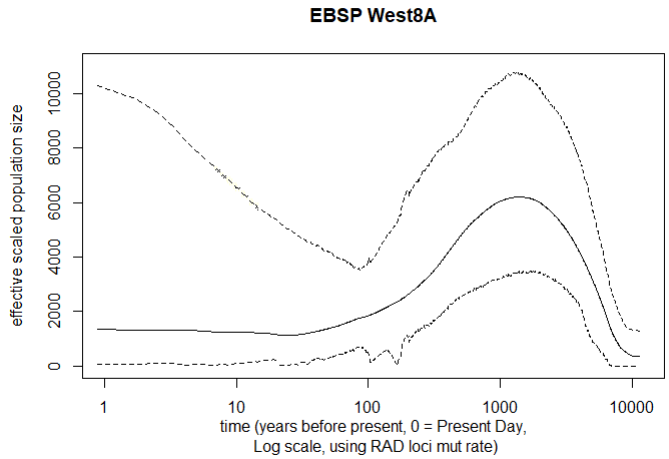



B)

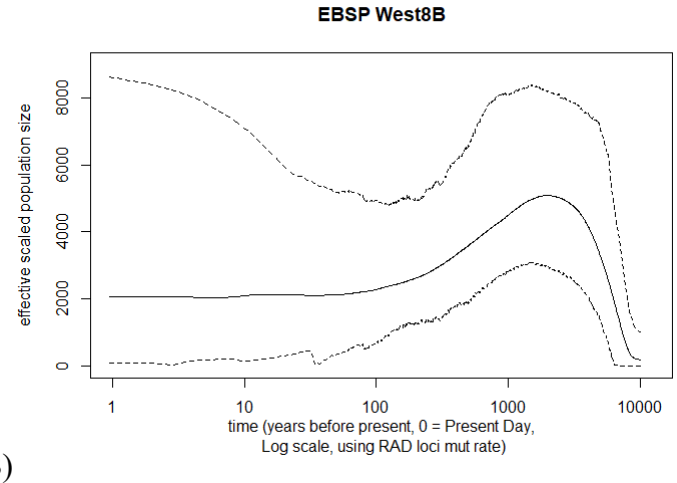



C)

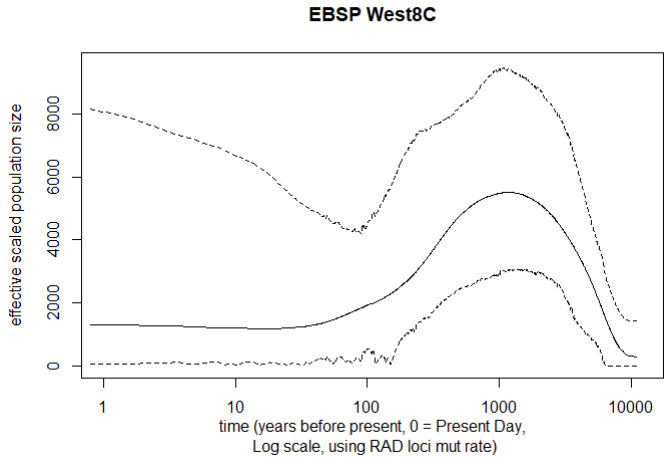
Figure 2.13. Extended Bayesian Skyline Plot (EBSP) outputs for eastern Australian populations of *Gymnorhina tibicen*. Each analysis was run using BEAST v2.5 using 50 randomly selected loci with 4-6 SNPs each.




A)

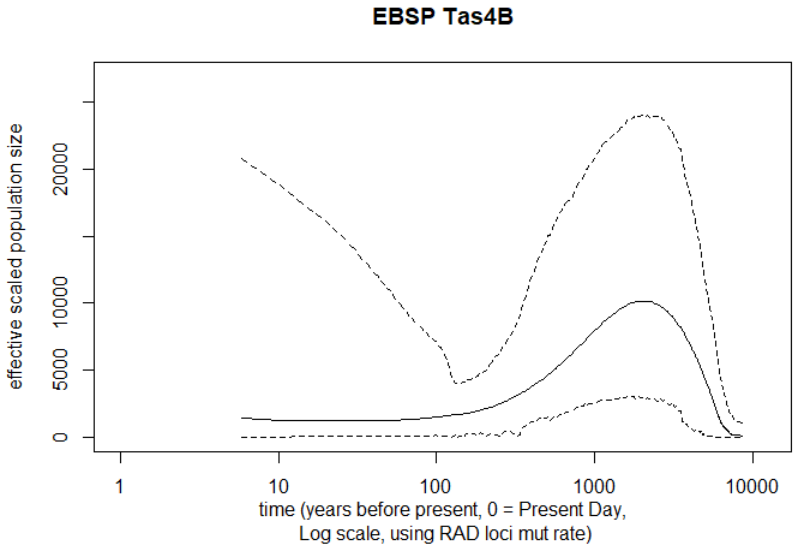



B)

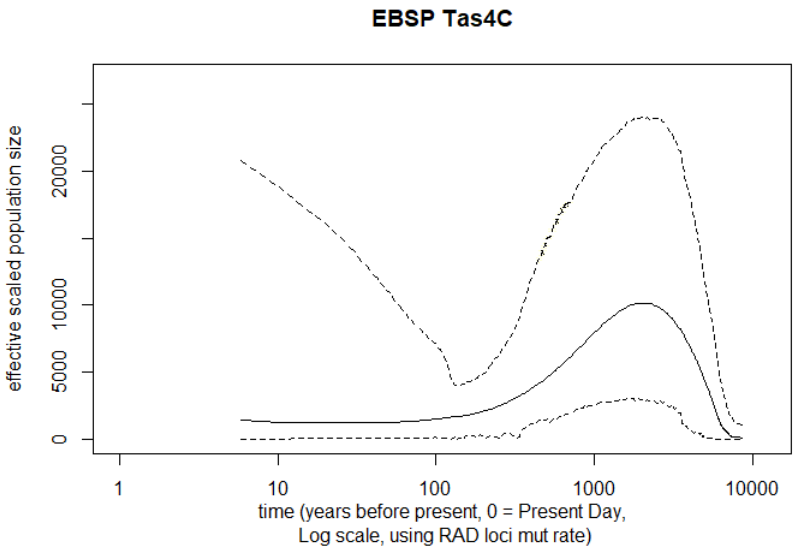



C)

1235 Figure 2.13 Extended Bayesian Skyline Plot (EBSP) outputs for western Australian populations of *Gymnorhina tibicen*. Each analysis was run using BEAST v2.5 using 50 randomly selected loci with 4-6 SNPs each.




A)




B)

Figure 2.14 Extended Bayesian Skyline Plot (EBSP) outputs for Tasmanian populations of *Gymnorhina tibicen*. Each analysis was run using BEAST v2.5 using 50 randomly selected loci with 4-6 SNPs each.

## 2.6 Discussion

### *2.6.1 Population genetic variation*

We have successfully obtained a genomic dataset for *Gymnorhina tibicen* that covers 29,406 SNPs across 15,345 loci. Exploratory analyses of the RADseq dataset revealed population structure between eastern and western mainland sites, Tasmanian sites and the mainland, and greater differentiation among western sites than among eastern. As nearly thirty thousand loci were used in this analysis, a great amount of variation must be accounted for, hence the relatively low explanatory power of the Principle Component Analysis. Despite this, the pattern observed here is roughly in agreement with that found in earlier studies (Toon et al., 2007), with south-western and south-eastern mainland populations exhibiting clear differentiation. Tasmanian populations possessed a greater number of private alleles than any mainland group. The Hobart samples in particular were more differentiated both from one another and the rest of the dataset. As this site represents the southern limit of *G. tibicen*'s distribution, it could be hypothesized to be a recent expansion followed by isolation from gene flow.

The clear differentiation between the western and eastern mainland sites, as well as between mainland Australia and Tasmania, was expected. Geographic barriers such as the Eyrean, Swan, Nullarbor and Bassian barriers have all been hypothesised to be critical in the formation of contemporary patterns of diversity for many Australian species, including many birds (Schodde and Mason, 1999, Keast, 1961, Ford, 1987, Dolman and Joseph, 2015, Joseph and Omland, 2009), with vicariance between eastern and western mainland populations observed across many taxa (Neaves et al., 2009, Cooper, 2003, Dubey and Shine, 2010, Guay et al., 2010). In many cases, the formation of these barriers is linked to intense and

widespread aridification that cause contraction and fragmentation of the mesic zones that are more suitable for many species (Byrne et al., 2011). The Bass strait, which has been linked to the mainland as recently as 8000ybp (Blom and Alsop, 1988), is also a known barrier to dispersal (McKinnon et al., 2004, Dawson, 2005). In the case of *Gymnorhina tibicen,* it appears that the Nullarbor arid barrier, as well as the Bass Strait, has had a significant influence on the present-day population structure of the species. This reinforces patterns observed in previous studies of *G. tibicen* using mitochondrial and microsatellite DNA markers (Toon et al., 2003, Toon et al., 2007), and similar patterns observed in the closely related genus *Cracticus* (Kearns et al., 2014).

The observed pattern of increased population structure among western mainland populations is echoed by similar patterns in some other Australian species (Rix et al., 2015), including spiders (Cooper et al., 2011), frogs (Driscoll, 1997) and plants (Hopper, 1979), but many more vagile species (namely birds and some mammals) do not show signs of population subdivision across southwestern Australia (Kearns et al., 2009, Joseph and Wilke, 2006, Guay et al., 2010). Where population subdivision has been observed in bird species within southwest Australia, it has often been attributed to the fragmentation of mesic habitat in the region (Toon et al., 2010, Donnellan et al., 2009, Guay et al., 2010). The ability and tendency to disperse across stretches of unsuitable habitat may be a common characteristic that these more structured species lack. In *G. tibicen* specifically, Baker et al. (2000) previously used mtDNA to identify higher levels of natal philopatry in southwestern populations than elsewhere, with lower levels of gene flow than among eastern populations. These life history traits may tentatively explain the contrast between the more structured southwestern populations of *G. tibicen* and other birds, as well as differences in habitat preference such as dependence on groundwater availability.

Of interest is the slight differentiation of Albany and Phillip Island from their neighbouring sites, as seen in the PCA and LEA results. Both show signs of slight admixture with their corresponding mainland cluster, but their differentiation from other sites in the same region trends in opposite directions, with Albany individuals showing greater similarity to the rest of the dataset, and Phillip Island being more differentiated. Albany is geographically closest to the Eastern sample sites, while Phillip Island is technically separated from the mainland by less than 1km of water, which may discourage dispersal to and from the mainland. A possible explanation for the admixture in Albany individuals is the presence of some historical gene flow from before the two regions were separated, and the greater philopatry and structuring among western populations has allowed 'eastern' coancestry to persist in the Albany population alone.

### *2.6.2 Demographic History*

Initial attempts at using the Extended Bayesian Skyline method to infer historical population size did not produce a clear demographic signal during preliminary tests. I suspect this was due to the confounding effect of population structure and sampling design on the skyline plot, as it has been demonstrated that results of Bayesian Skyline runs can show false signals of decline if the assumption of panmixia is violated (Heller et al., 2013, Orozco-terWengel, 2016). This may be a fault with the sampling design of this analysis, as sequence data from the same number of individuals spread over a much larger geographic area would not be so strongly structured. Future analysis of demographic history would benefit from a sampling regime more representative of the total genetic variation across each cluster's range.

While the timing inferred from the EBSP analyses is more recent than anticipated, the overall demographic changes and their timing relative to one another somewhat aligns with expectations. It has been inferred as part of prior studies that eastern populations of *Gymnorhina tibicen* experienced widespread population expansion after the Last Glacial Maxima, dispersing from refugia situated in the southeast of the Australian mainland (Toon et al., 2007). Many warm climate-adapted species globally show similar signs of range withdrawal into glacial refugia during ice ages, before expanding again during interglacial periods (McKinnon et al., 2004, Provan and Bennett, 2008, Weir et al., 2016).

Conversely, western populations of *G. tibicen* have been thought to be more stable during the post-glacial period, and appear to have expanded and subsequently diminished in population size, a pattern which is repeated in the Tasmanian group. If this expansion coincided with the Last Glacial Maximum, lowered sea levels may have enabled western populations to expand over the Sahul shelf (Toon et al., 2017). Population expansion during glacial periods is a phenomenon observed in many arid-adapted species in Australia (Byrne et al., 2008, Fujita et al., 2010, Kearns et al., 2014). The contrast in demographic history between two populations of the same species may have to do with the relative suitability of available habitat after the last glacial period on either side of the continent, or it may be attributable to differing levels of adaptation to arid conditions between the groups. These patterns might be supplemented with ecological niche modelling approaches to better understand the conditions that have influenced the species' demographic history. An approach that includes modelling each of the three main subunits established in this chapter (eastern, western and Tasmanian) separately may be particularly useful to take into account local adaptive responses (Smith et al., 2019). Presence/absence data for *G. tibicen* is available across the species' distribution through the

Atlas of Living Australia platform, from both field sightings and museum specimens, that would serve as a useful starting point for these analyses (Belbin, 2011).

# CHAPTER 3: COMPARISON OF SINGLE-GENE AND WHOLE GENOME PHYLOGENIES OF *GYMNORHINA TIBICEN*

## 3.1 Abstract

Studies of the patterns of genetic structure and connectivity among populations of Australian birds are often based on one or few genetic loci, as is the case with the Australian magpie (*Gymnorhina tibicen*). In this chapter, I have attempted to construct whole mitochondrial genomes of *G. tibicen* from short read sequencing data using multiple tissue types, and compare the resulting phylogenetic trees constructed from different genes and whole mitogenomes. I also incorporate newly available samples into our understanding of the phylogeographic structure of *G. tibicen*. With this mitogenomic data, I have investigated several questions: how strongly supported are the eastern and western Australian clusters identified previously from single genes? How do phylogenies inferred from different mitochondrial genes and whole genomes compare to one another? And where do previously unsampled areas fall in the greater phylogeny of *Gymnorhina tibicen*? Relatively fresh liver tissue produced the greatest fraction of mitochondrial reads from whole DNA extraction, with five complete mitogenomes assembled, while DNA derived from frozen blood produced near-complete genomes. The results of the phylogenetic analysis support the existence of eastern and western mainland clades of *G. tibicen*, with individuals from Tasmania and Flinders Island most closely related to the eastern mainland. Of additional interest is the placement of an individual from central Western Australia in the eastern cluster, which suggests that the 'eastern' group may extend further into the interior than its previously identified limit of Alice Springs. Finally, I provide several recommendations to build upon

the findings of this study in the form of additional sampling locations and more suitable tissue specimens for mitogenomic assemblies.

## 3.2 Introduction

### *3.2.1 Biogeography of Australia & New Guinea*

Similar patterns of genetic divergence across the Australian mainland, Tasmania and the Pacific Islands have been observed in a number of different taxa. Generally, these are attributed to the presence of common geographic barriers that limit dispersal and generate vicariance. These range from arid barriers, as in the case of the Nullarbor and Carpentarian barriers in the south and north of the continent, which are thought to have created deep East-West divergences in many Australian species (Ford, 1982, Dolman and Joseph, 2015, Toon et al., 2007, Crisp and Cook, 2007), inland lakes, such as the Eyrean basin, and historical land bridges such as those that existed across Bass Strait and the Sahul Shelf (Dolman and Joseph, 2015, Dawson, 2005, McKinnon et al., 2004). As recently as the Pleistocene, a savannah-dominated land bridge connected the landmasses of Australia and New Guinea and made gene flow possible, until sea levels rose at the end of the last glacial period approximately 16kya (Byrne et al., 2008).

Many bird species are shared between Australia and New Guinea (Bishop, 2006, Beehler et al., 1986, Joseph and Omland, 2009, Bowman et al., 2010). However, not all species with Australasian distributions exhibit the same patterns of divergence. For instance, populations of the pied butcherbird (*Cracticus nigrogularis*) from New Guinea and Cape York are closely related to one another, and together the two groups are thought to be sister to populations from the Kimberley region (Kearns et al., 2014). A similar pattern of gene flow across the Torres Strait has been observed in the little shrike-thrush (*Colluricincla megarhyncha*) (Deiner et al., 2011). This contrasts with signatures of gene flow observed in the blue-winged kookaburra (*Dacelo leachii*) and the Australian magpie (*Gymnorhina tibicen*), both of which

have New Guinean populations most closely related to those in the Kimberley region of North Western Australia, with past connections thought to have taken place along the Arafura shelf to the west of the Carpentaria Basin (Toon et al., 2017, Dorrington et al., 2020). These conclusions were drawn from a limited number of nuclear and mitochondrial loci, however, and analysis using a broader range of genes may provide further insights.

### *3.2.2 Use of mitochondrial sequence data in phylogeographic studies*

Phylogeographic analysis of mitochondrial sequence data has allowed researchers to make inferences about historical range expansions, contractions, divergences and bottleneck events (Joseph and Omland, 2009, Dolman and Joseph, 2015). Historically, the mitochondrial control region in particular has been used, as it is generally assumed to be the most rapidly evolving portion of the mitogenome – severalfold times faster than other mitochondrial genes (Aquadro and Greenberg, 1983). This heightened mutational rate, combined with the well-understood structure of the molecule and the predictable maternal mode of inheritance for the organelle, makes it a valuable tool for phylogenetic, phylogeographic and population genetic studies. However, estimates of the exact rate vary, some studies providing estimates much closer to that of Cytochrome *b* (Zink and Blackwell, 1998), with the rate of mutation per million years ranging from 2.8% in humans (Cann et al., 1984) to 20.8% in a study on snow goose (*Chen caerulescens*) (Quinn, 1992). Rates estimated from one mitochondrial marker are often extrapolated to other markers, but studies suggest that rates of evolution of commonly used genes are not necessarily reflections of rate variation in other parts of the genome (Nguyen and Ho, 2016). The relationships between rates of evolution of different genes can also vary considerably among taxa. In Aves, for example, it has been demonstrated that the commonly used genes, Cyt*B* and *CO1,* exhibit considerably different patterns of rate

variation across different bird lineages (Lavinia et al., 2016). As a consequence of this variation, studies that use single genes or a small number of genes often produce phylogenetic trees with different branching orders and poor support values (Tobe et al., 2010, Kelly et al., 2003, Rokas et al., 2003), a problem which is exacerbated below the species level (Lavinia et al., 2016). Increasing the number of genes used, or utilizing whole genomic datasets, are two methods by which these limitations can be addressed.

### *3.2.3 Australian magpie mitochondrial DNA studies*

Previously, studies using mitochondrial data from *Gymnorhina tibicen* have provided insights into broad population genetic structuring, identifying several major groupings and possible refugia during the last glacial maxima. Early studies used variation in the control region to identify disjunctions between populations in the eastern and western mainland and compared levels of population genetic structuring within these clusters (Toon et al., 2003, Baker et al., 2000, Hughes et al., 2001). Subsequent studies incorporating mtDNA and microsatellite data reinforced the presence of non-overlapping groups in east and west mainland Australia, though the boundaries of these groups differed between nuclear and mitochondrial markers. Estimates using single mitochondrial genes placed the date of their divergence at approximately 36kya, and also estimated the divergence between Tasmanian and mainland populations of *G. tibicen* to have taken place 16kya (Toon et al., 2007). A more recent study, also by Toon et al. (2017), incorporated samples of the putative subspecies *G. tibicen papuana* from New Guinea into the existing mitochondrial dataset as part of an assessment of the common evolutionary history between Australian and New Guinean biota. The finding that *papuana* was most closely related to northern and northwestern subspecies of *G. tibicen* supported earlier observations that were based solely on morphology (Black, 1986), and echo

findings in some species whose distributions span the Torres Strait (Dorrington et al., 2020). To date, these studies have used a relatively low number of genes compared to what is feasible using current sequencing technology.

The search for increasingly high-quality genomic data has focused on whole mitochondrial sequencing for many study species. With regard to Australian magpies, a 2019 study attempting to reconstruct the mitochondrial genome of *G. tibicen* in its entirety from museum eggshells was moderately successful, but could not confidently reconstruct the D-loop due to the repetitive nature of some sections, which creates issues when assembling genomes from short reads (Grealy et al., 2019). Alternative approaches to mitogenome assembly and higher coverage of sequence data may address these limitations.

### *3.2.4 Project aims*

This project aims to provide new information on genetic relationships among populations of *G. tibicen* in mainland Australia, Tasmania and New Guinea. This will ideally build on the findings of Toon et al. (2017), which were based only on elements of the mitochondrial d-loop and cytochrome b. Several newly available samples from Papua New Guinea, central Western Australia, and two new Tasmanian sites from museum collections and recent fieldwork have made it possible to incorporate these locations into our understanding of the biogeographic history of *G. tibicen*. Newly available samples from Lake Carnegie, located close to the centre of WA, are of particular note. Most available tissue samples from the western populations of *G. tibicen* are coastally distributed, and no inland samples west of Alice Springs have previously been included in population genetic studies.

Of additional interest is whether relatively 'fresh' DNA from more recently collected specimens can provide more, or higher quality, sequencing reads, and whether this in conjunction with novel approaches to genome assembly can produce a more complete control region than those generated by museum eggshell specimens in prior studies (Grealy et al., 2019).

The aims of the study were as follows:

i) To incorporate new sample sites & loci into a comparison of relative genetic distances among populations and relative variation within populations of *G. tibicen*. Samples from Lake Carnegie in WA and Flinders Island, Tasmania, have not previously been represented in

phylogeographic studies of *G. tibicen*. Broad eastern and western groups have been identified in prior studies of the species, but the relative levels of genetic variation may vary among genes. The species is hypothesised to have persisted in south east and south west mainland refugia during the last glacial maximum (ending approximately 19,000 years before present), before dispersing outward across most of the continent (Toon et al, 2007). Differences in patterns of genetic variation between the eastern and western clusters will provide insights into the biogeographic forces that shapes each population.

ii) To compare phylogenetic trees generated from whole mitochondrial sequence data and individual genes, specifically the control region and cytochrome *b*. Current understanding of the relationships between populations of *G. tibicen* has been based on only a few hundred base pairs of the control region. By comparing phylogenies based on mitochondrial genes, we can assess whether or not the control region alone accurately represents the relationships between populations.

iii) To investigate whether the eastern mainland cluster identified in previous studies (Toon et al., 2007) extends even further west. At present, samples from Alice Springs in the Northern Territory have been suggested to be part of a group distributed mainly across the eastern half of the mainland.

iv) To investigate the relationship of Flinders Island samples to Tasmanian and eastern mainland populations. Due to the proximity of the Tasmanian mainland and relative ease of dispersal southwards, it is expected that Flinders Island and other Tasmanian samples would form a clade separate to those found on the mainland.

v) To reconstruct a complete mitochondrial genome for *G. tibicen.* While previous research has produced a nearly complete mitogenome (Grealy et al., 2019), the D-loop could not be verified as completely intact. By using alternative sequencing & assembly methods, as well as fresher organ tissue instead of museum eggshell samples, I hope to produce a complete, circularized genome with relatively high coverage.

## 3.3 Methods

### *3.3.1 Sampling Design*

While it would be ideal to implement a sampling regime like that used in previous population-level genetic studies, I was restricted in the number of samples that could be submitted for sequencing. This restricted the sampling design for the project, and so samples were selected to maximise geographic coverage and incorporate newly available sample sites. A total of 27 specimens of *G. tibicen* were selected from sites around mainland Australia, Tasmania and New Guinea (figure 1). Of these, 20 were blood samples collected as part of other population genetic studies of Australian magpies between 1994 and 2007 (Baker et al., 2000, Toon et al., 2007), with another collected in 2017 as part of a different project during this candidature (Chapter 2). The remaining six samples were provided by the Australian National Wildlife Collection, and included two sites from Western Australia, three sites in South Australia and a specimen from Papua New Guinea. Site coordinates and ANWC IDs are available in Table A1. Additionally, two blood samples from pied butcherbird (*Cracticus nigrogularis*) specimens were included as outgroup samples for the phylogenetic analyses.

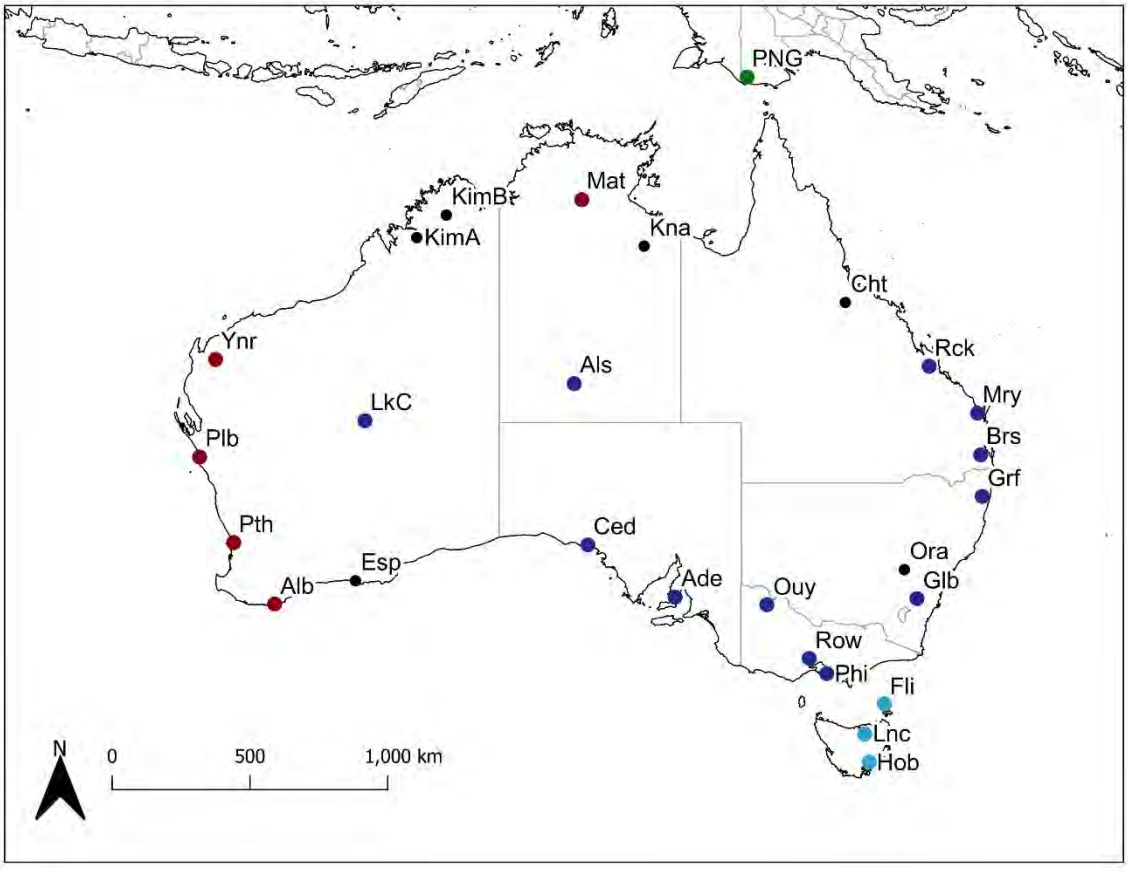



Figure 3.1 Map of sampling locations and IDs for *Gymnorhina. tibicen.* Red, green, light blue and dark blue sites represent clades recovered from phylogeographic analysis, while black sites represent sampling locations that did not produce enough reads to assemble full or partial genomes. Full sample IDs and location information are available in Appendix I**.**

### *3.3.2 Laboratory Methods*

DNA extraction was done using a protocol developed from frameworks provided by earlier studies (Prince, 2015, Etter et al., 2011, Catchen et al., 2013). Blood samples were suspended in a solution of blood lysis buffer, in a rough 2:1 ratio of buffer to blood. 100-150µl of each buffered sample was added to a 1.5mL Eppendorf tube. As some samples were collected into lysis buffer over a decade prior to this project, additional mechanical agitation was necessary to break apart congealed samples and obtain sufficient concentrations of high-quality DNA. To this end, Phosphate-buffered saline (PBS) solution was then added to each sample to a total volume of 500µl, and the samples were then bead-beaten with a Tissue Lyser II (QIAGEN), using one 3mm tungsten carbide bead (QIAGEN) per sample, at 30 beats/second for 10 seconds. Wherever possible, clotted blood (increased coagulation being a side effect of suspension in lysis buffer) was drawn from the available samples, as clots produce higher yields of DNA than unclotted samples.

The bead-beaten samples were transferred to new 1.5mL Eppendorf tubes, and samples were digested for a minimum of 8 hours at 55°C in a heat block until all coagulated blood was broken down. 200µl of 2M guanidine hydrochloride, 200µl of 100% ethanol and 100µl of precipitation buffer (4M Ammonium Acetate) was then added to each sample, before resting for an additional 15 minutes at room temperature. Each sample was then transferred to an EconoSpin column (Qiagen) and centrifuged at 16,000g for 15 minutes. The resulting flow-through solution was discarded from the collection tube, and 500µl of binding buffer (2M GuHcl, 75% Ethanol) was added to each column. The samples were centrifuged again (16,000rcf, 15 minutes), discarding the flow-through solution, before 500µl of wash buffer (80% Ethanol, 1M Tris) was added to each tube and centrifuged at the same settings, again

discarding the flow-through. The wash buffer step was repeated a second time, and the spin column was moved to a 1.5mL Eppendorf tube. 80µl of elution buffer was added directly to the membrane of each column and allowed to soak for 30-60 minutes before being centrifuged at 6,000rcf for 1 minute. Organ tissue samples had DNA extracted using a similar protocol, with the bead beating element removed.

The eluted DNA samples were visually assessed for quality and concentration through Agarose Gel Electrophoresis. 2-3µl of sample was mixed with 1µl of Gel Loading Dye and run on a 1.2% Agarose gel at 80V for 1 hour before visualisation on a GelDoc XR+ Imager. Once the quality of DNA was confirmed, the samples were subsequently assessed for DNA concentration using a Qubit Fluorometer. DNA samples were sent to the Australian Genomic Research Facility (AGRF) and run on an Illumina sequencing platform. Data were generated as 150bp paired-end reads and the sequencing output + quality scores were returned as a compressed FastQ sequence file for each individual.

*3.3.3 Data assembly and quality checks*

The program NOVOplasty (Dierckxsens et al., 2017) was used to assemble the mitochondrial genome de novo from the paired-end reads. Most settings for mitochondrial genomes were left at the default, with the K-mer setting reduced to 33 to tolerate slightly shorter overlaps between reads. For the seed sequence, a fragment of cytochrome b from a *G. tibicen* specimen available on GenBank (accession no. AF197867) was used. Samples that failed to assemble whole circular genomes were mapped to the whole mitogenome with the highest coverage in Geneious 2020.1.1 (https://www.geneious.com). To verify whether the permitted overlap between reads was sufficient to cover short repetitive sequences, a series of test

assemblies were run on individual samples, setting the minimum K-mer length at 23, 33, 45 and 55. Provided there were no discrepancies in these tests, any completely assembled genomes were submitted to the MITOS web service (Bernt et al., 2013) for annotation. The returned annotations were inspected for appropriate start/end regions for each gene, and the annotated genomes were then compared to that of several closely related species (*Corvus splendens, Artamus cinereus, Cracticus nigrogularis*), as well as the previously published *G. tibicen* partial mitochondrial genome from Grealy et al. (2019), to verify the completeness of the genomes.

### *3.3.4 Phylogenetic analysis*

Analyses were run on the control region, cytochrome *b*, CO1 and ND2 for comparison with the findings of earlier studies (Toon et al., 2017). Each alignment consisted of only unique haplotypes with greater than 80% of bases classified as high quality (with only 4 samples having a HQ% of <90). *Cracticus nigrogularis* was included as an outgroup and used to root the trees. Neighbour-joining trees were constructed within Geneious to visually assess the relationships between haplotypes, and RAxML was used for a maximum likelihood approach to tree estimation. Mutation models were determined using the corrected Akaike information criterion in jModelTest (Guindon and Gascuel, 2003, Darriba et al., 2012). For the individual genes, the substitution model GTR+GAMMA+I was used. A maximum likelihood tree was also constructed using the complete assembled mitochondrial genomes, for which a GTR+GAMMA model was used. 1000 bootstraps were run to estimate support for each branch.

### *3.3.5 Comparing variation within & among clades*

To compare genetic variation within and among the clades identified in the phylogenetic analysis, a matrix of genetic differences was constructed for the control region, CO1, and cytochrome B, as well as the complete mitogenome. The number of base pair differences, and percentage of the total sequence length that was dissimilar between each pair of individuals, were used as measures of divergence. The average difference between individuals within the same clade was calculated, as was the average difference between individuals of the three major clades of *G. tibicen* (Eastern, Western and Tasmanian). Approaches for measuring variation within the dataset were limited, due to the low number of samples and uneven distribution of samples across the identified clades.

## 3.4 Results

### *3.4.1 Mitogenome assembly*

Raw reads from the extracted samples ranged from 2.8 million to 7.8 million reads per individual, with a mean of 4.98 million reads per sample. Of the initial NOVOplasty assemblies, five samples were successful outright (Table 1), with the percentage of raw reads used ranging from 0.11-1.41%, and an average of 98% of bases having quality scores of Q30 or greater. Coverage for the *G. tibicen* samples was very high, with a mean coverage of 653x. The size of the assembled mitogenomes ranged from 16,866 - 16,867bp, slightly smaller than the 16,874bp assembly of Grealy et al. (2019). Variation in the size of the assembled mitogenomes was traced back to variation in the length of a single nucleotide repeat sequence located in the control region. Additionally, one of the two *Cracticus nigrogularis* samples successfully assembled a complete mitogenome 16,893bp in size, with a mean coverage of 14.8x. This sample was retained for use as an outgroup in the phylogenetic analysis. Running the assemblies in NOVOplasty with increasingly demanding overlap thresholds did not produce any variation in the size of the assembled contigs, suggesting that short repeats were not being omitted and truncating the length of the contig.

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Table 3.1 Statistics for the mtDNA assemblies that produced complete circularized genomes for *G. tibicen*. Shown are the number and percentage of reads used in the assembly, the mean coverage and the number of sites recovered for each sample (genome size).

| Sample ID | Sample Location | Raw Reads | Aligned Reads | Assembled Reads | % mtDNA | Mean coverage (x) | Bases recovered (bp) |
|---|---|---|---|---|---|---|---|
| NM05_YNR | Yanarrie, WA | 7275796 | 65534 | 50776 | 0.9 | 583x | 16867 |
| NM06_LKC | Lake Carnegie, WA | 10333156 | 86774 | 66340 | 0.84 | 772x | 16866 |
| NM22_ADE | Adelaide, SA | 8606292 | 121724 | 95894 | 1.41 | 1082x | 16867 |
| NM24_FLI | Flinders Island, TAS | 5664342 | 43014 | 33056 | 0.76 | 383x | 16866 |
| NM27_PNG | Papua New Guinea | 7443088 | 8428 | 6498 | 0.11 | 75x | 16867 |

### *3.4.3 Phylogenetic analysis*

Both Neighbour-Joining and RAxML trees produced patterns demonstrating clear genetic divergences between eastern, western and Tasmanian clades of *Gymnorhina tibicen*, with strong support values for each of the major clades (Figure 3.2). The sample from Papua New Guinea most closely associated with individuals from the western mainland, while the Tasmanian clade was sister to the eastern. Maximum likelihood trees for the control region, CO1 and cytochrome *b* produced similar major clades to the complete mitogenome (Figure 3.3) but did not exhibit much structure within clades, apart from cytochrome *b* (Figure 3.3C). Both *CO1* and *CytB* strongly support the existence of the Tasmanian clade and resolve the western clade with greater clarity than the d-loop. Of the specimens from previously unsampled regions, Lake Carnegie in WA was placed within the 'eastern' clade, and Flinders Island with the Tasmanian samples.

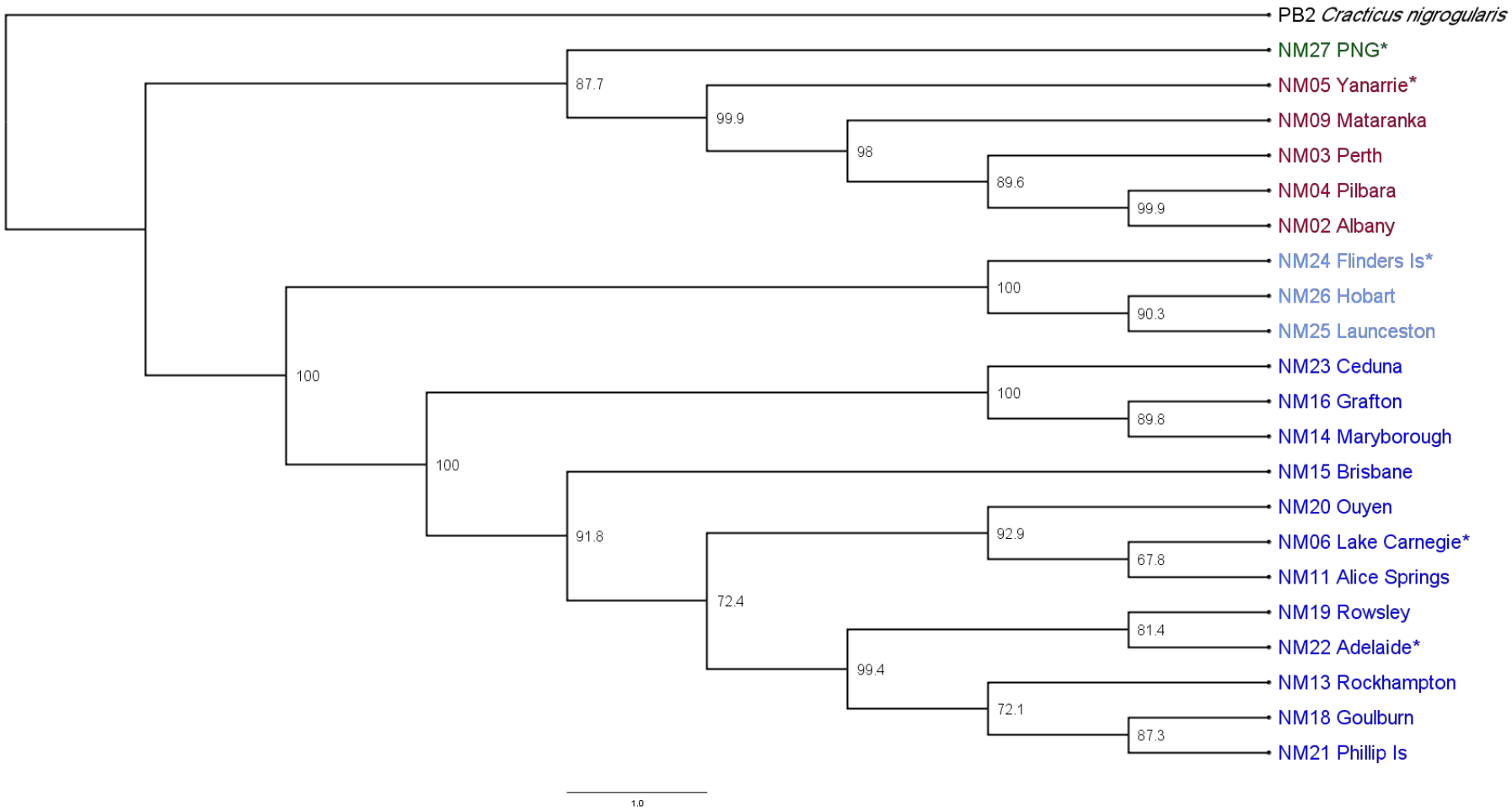
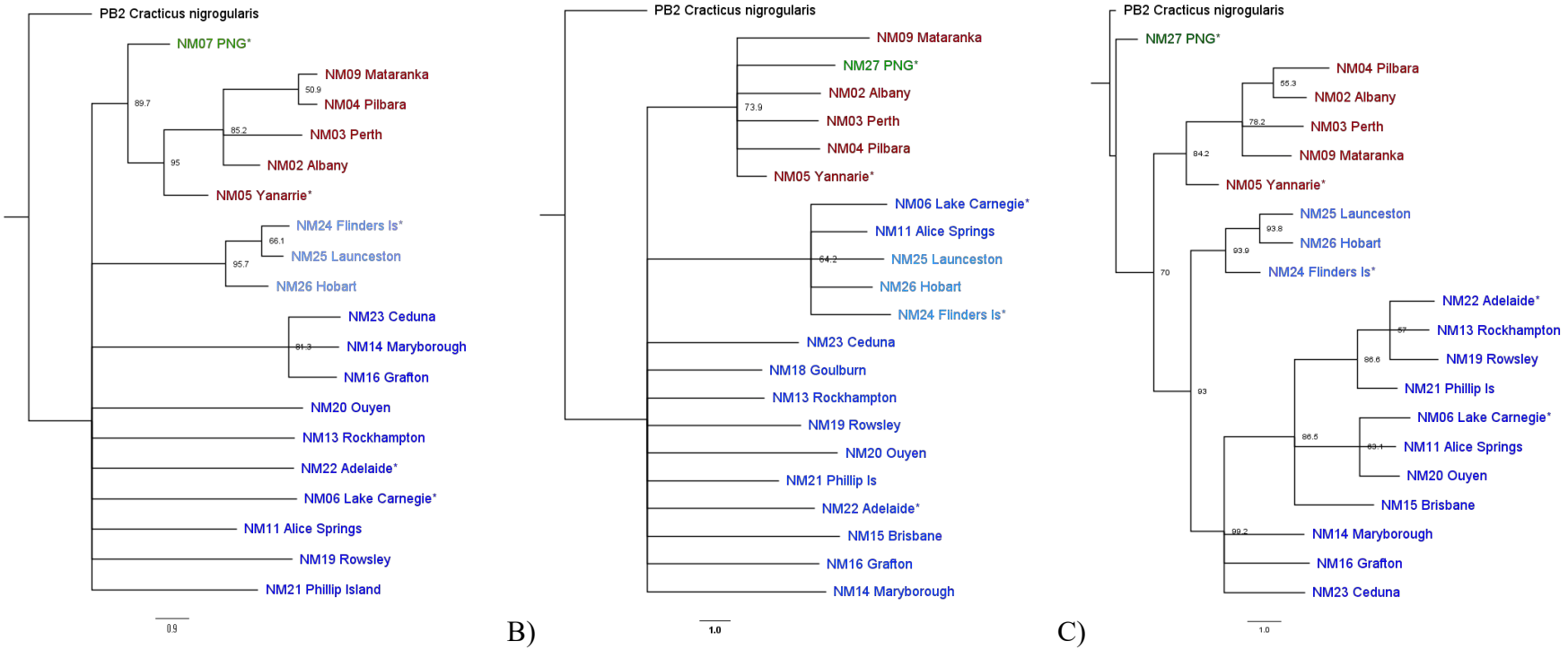



Figure 3.2 Phylogeny of *Gymnorhina tibicen* based on complete and near-complete mitochondrial sequence data. Calculated with maximum likelihood analysis in RAxML, node values shown are support values. Colours represent western (red), eastern (dark blue) and Tasmanian (light blue) clades, with Papua New Guinea in green. *G. tibicen* samples that were assembled into complete circularized genomes denoted with asterisks (*). *Cracticus nigrogularis* outgroup is shown in black.




A) B) C)

Figure 3.3 Phylogenies of *Gymnorhina tibicen* estimated with maximum likelihood analysis implemented in RAxML.Trees are based on A) CO1, B) partial sequence from the D-loop (539bp of regions 1 & 2), and C) Cytochrome B. Node values shown are support values. Haplotypes are colour coded to represent western (red), eastern (dark blue) and Tasmanian (light blue) sites. *Cracticus nigrogularus* outgroup is shown in black. Haplotype in green is the *G. tibicen papuana* specimen from the ANWC.

*Variation within and among clades*

The matrices of genetic differences among clades for each gene of interest displayed similar trends. The western clade contained the most genetic variation and the Tasmanian clade exhibited the least (Table 2), with the Tasmanian samples more similar to the eastern mainland clade than the western clade.

Of the individual genes compared, the mitochondrial control region exhibited the greatest differentiation within clades. One exception to this trend was the greater variation of cytochrome *b* in the Tasmanian clade, but this can be attributed to the small number of samples being compared. CO1 showed the least difference within clades of the genes compared here.

Table 3.2 Mean genetic similarity of *Gymnorhina tibicen* mtDNA sequences. Calculated using complete mitochondrial genomes, the mtDNA control region, *CO1* and *Cytochrome B.*

| | Similarity within clade (%) | | | Similarity between clade (%) | | |
|---|---|---|---|---|---|---|
| **Sequence** | **Eastern** | **Western** | **Tasmanian** | **East v West** | **East v Tas** | **West v Tas** |
| **Whole mitogenome** | 99.550 | 99.352 | 99.702 | 98.049 | 99.206 | 98.044 |
| *Control region* | 98.973 | 99.168 | 99.892 | 98.160 | 99.310 | 98.337 |
| *CO1* | 99.775 | 99.770 | 99.799 | 98.350 | 99.447 | 98.226 |
| *CytB* | 99.583 | 99.472 | 99.679 | 97.586 | 98.616 | 97.552 |

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## 3.5 Discussion

### *3.5.1 Sequencing and genome assembly*

The five successful *G. tibicen* mitogenome assemblies were derived from DNA extracted from liver tissue provided by the ANWC, while the remainder were blood samples collected over a span of >20 years. The average number of raw reads obtained for individuals that were successfully assembled into whole mitogenomes was lower than that in the unsuccessful assemblies (3.98 million reads as opposed to 5.2). While the blood samples have undoubtedly undergone a large number of freeze-thaw cycles in the past 20 years, studies have suggested that blood samples, particularly avian blood, can go through multiple cycles of freezing and thawing without any reduction in quality (Seutin et al., 1991). However, mtDNA has been shown to be much more sensitive in this regard, with the proportion of mtDNA to nuclear DNA diminishing rapidly when thawed to room temperature (Berger et al., 2001). Additionally, the mtDNA copy number is significantly lower in blood tissue than other organs, often by an order of magnitude (Laubenthal et al., 2016, Bogenhagen, 2012, Barazzoni et al., 2000). These compounding factors make it plausible that the fraction of mitochondrial DNA in the blood samples was initially too low for sufficient high quality reads to be assembled in most samples, despite the greater overall number of reads obtained by sequencing.

Testing increasingly demanding overlap thresholds during assembly did not produce any variation in the size of the final assembled contigs, which suggests that short repetitive sequences were not interfering with assembly by truncating the sequence. It has been highlighted in other studies that the Illumina sequencing technology has problems with high rates of error after long single nucleotide repeat (SNR) stretches, making the subsequent

reads unreliable (Treangen and Salzberg, 2011, Ross et al., 2013). NOVOplasty attempts to address this issue by selecting the most frequent recurrent length, which has been observed to be the most accurate (Dierckxsens et al., 2016). Taking these points into consideration, I tentatively suggest that the circularised sequence data from this project do represent complete mitochondrial DNA genomes of *Gymnorhina tibicen*.

To resolve the uncertainty regarding the mitochondrial control region and confirm that the assembled genomes are accurate, a targeted sequencing approach may be required, in which the control region and adjacent genes are amplified using specifically designed primers in conjunction with a proofreading enzyme. Studies have demonstrated the suitability of this approach where low coverage of the control region or ambiguous read lengths make accurate sequencing using assemblies of short reads difficult (Bronstein et al., 2018). Long-read sequencing approaches have also been used to assemble whole mitogenomes from a range of sample types, including low-yield or poorer quality sources such as eDNA, with a high degree of accuracy (Baeza, 2020, Deiner et al., 2017). This emerging technology has shown suitability for obtaining sequence data for structurally complex mtDNA regions (Kinkar et al., 2019), and shows promise for the resolution of the control region of *G. tibicen*.

### *3.5.2 Phylogenetic analyses*

The close association between the Papua New Guinea sample and those from the western mainland of Australia is consistent with the findings of earlier studies (Toon et al., 2017). As suggested in the aforementioned study, this and a similar pattern of connectivity between Cape York and Papua New Guinea in the black-backed butcherbird (*Cracticus mentalis*) (Kearns et al., 2014) both reflect the broad connective habitat that once spanned the Sahul

Shelf, a region of savannah and sparse woodland that was lost after the Last Glacial Maximum (LGM), approximately 20,000 years ago. The reason for the different patterns of connectivity on the East and West sides of the Sahul Shelf is yet to be discerned and depends on continued study of the environmental history of the region. However, it is possible that the habitat preferences of the study species are at least partially responsible for contemporary patterns of connectivity, with *Gymnorhina tibicen* having a greater preference for open habitat and terrestrial foraging than many of its relatives within *Cracticus* (Kearns et al., 2013, Schodde and Mason, 1999).

As predicted, the Flinders Island sample consistently grouped with those from Tasmania rather than the mainland. A similar relationship has been observed in other animal taxa, including reptiles and marsupials (Ng et al., 2014, Cooper et al., 2018). This close relationship is to be expected given the relative ease of dispersal southward, as at its closest point Flinders Island is only 50km from the island of Tasmania, with several large intervening islands, while the distance to the Australian mainland across Bass Strait is approximately three times greater across open sea.

In relation to the initial hypotheses concerning the placement of the specimen from Lake Carnegie, in central Western Australia, an earlier study suggested that the Eastern clade of *G. tibicen* extended as far inland as Alice Springs, NT (Toon et al., 2007). Placement of the Lake Carnegie specimen in the ML trees suggests that it too is part of the 'Eastern' clade, with a close relationship to the specimen from Alice Springs, some 1,200km to the east. While more extensive sampling would be required to make any broad generalisations about the biogeographic history of Western Australia, past studies have inferred a historically stable

population size and a greater degree of genetic structuring among western populations of *Gymnorhina tibicen* than eastern (Toon et al., 2007), as well as chapter 2 of this thesis. From this, it might be suggested that some aspect of the Murchison and Gascoyne bioregions makes them unsuitable habitat for *G. tibicen* and has hindered dispersal further inland for Western populations.

The Eyrean and Carpentarian barriers (Schodde, 1982) have been linked to genetic differentiation among populations of avian species closely related to *G. tibicen*, namely the butcherbirds (Kearns et al., 2010, Kearns et al., 2014) as well as more distantly related species (Joseph and Wilke, 2006, Dolman and Joseph, 2015, McLean et al., 2017), commonly creating clear genetic divergence between populations to the east and west of their respective barriers. The grouping of specimens from Ouyen, Alice Springs and Lake Carnegie into a monophyletic clade despite their considerable distance may be the result of historical dispersal inland, north of the Eyrean barrier. The Australian magpie is not particularly well adapted to arid environments and exhibits a close association with groundwater (Harrington, 2002, James et al., 1999). The increased abundance of artificial groundwater since human settlement of inland Australia is likely to promote dispersal of *G. tibicen* into areas they previously did not inhabit, or inhabited at much lower densities. A sampling design aimed at creating a population genetic dataset for sites such as Lake Carnegie and Alice Springs, as well as increased sampling resolution throughout the arid interior of continental Australia, would help shed light on the persistence of a widespread generalist such as *G. tibicen* in unfavourable conditions.

*3.5.3 Genetic variation within and among clades*

The broad trends in genetic variation within and among clades of *G. tibicen* are in line with findings from earlier population genetic studies of the species using microsatellites and control region sequence data (Toon et al., 2007). Across each individual gene that was analysed, as well as the complete mitogenome, the matrices of sequence similarity showed similar trends: The western specimens exhibited high levels of differentiation between one another, while the Tasmanian clade showed the least, and the Tasmanian clade was more similar to the eastern clade than the western. These findings are further supported by my own population genomic analysis using RADseq data, as in Chapter 2 (unpub. data).

The greater structure observed among western populations compared to those in the eastern mainland is part of a greater trend of relatively recent population subdivisions among both vertebrate and invertebrate taxa (Rix et al., 2015), although many more vagile species (namely birds) do not show signs of significant population subdivisions across southwestern Australia, (Kearns et al., 2009, Joseph and Wilke, 2006, Guay et al., 2010). Patterns observed in *Gymnorhina tibicen* could tentatively be attributed to differences in life history, with western populations being hypothesised to exhibit greater natal philopatry and restricted gene flow relative to eastern populations (Baker et al., 2000, Toon et al., 2007).

The control region was the most variable of the individual genes within clades, with the exception of *Cytochrome B* in the Tasmanian clade (though this may be an artefact of low sample size). *CO1* was the least variable of the genes analysed as part of this study.

*Considerations for future studies*

While the initial aims of the study have been met, the sampling design was subject to multiple limitations and could readily be expanded on. The location of samples was restricted to those already present in the laboratory collection from past studies and on loan from the Australian National Wildlife Collection. The number of samples included was also restricted by the available space on an Illumina sequencing run organised by other projects. There are a number of ways in which these factors might be addressed in future studies.

As mentioned, the copy number of mtDNA in a cell varies greatly across different tissue types (Bogenhagen, 2012), with a mean of ~200 in blood tissue and 2000-6000 in other tissues such as the liver, kidneys or heart (Laubenthal et al., 2016). Accordingly, if more time and resources could be devoted to sampling with the intent to produce complete mitochondrial genomes, it would be appropriate to use only museum tissue specimens from organizations like the Australian National Wildlife Collection, or to biopsy said tissues from deceased individuals in the field, prioritising organs with relatively high copy numbers of mitochondrial DNA such as the heart and liver. This approach would help mitigate the issues associated with lower quality assemblies derived from blood samples. To improve the proportion of mtDNA reads recovered for each sample during sequencing, a range of enrichment methods might also be implemented (Burgener and Hübner, 1998, Mascolo et al., 2019, Gould et al., 2015). By reducing the nuclear DNA content, any false positives created by nuclear mitochondrial sequences (numts) may also be avoided.

In order to clarify the distribution of the known clades of *Gymnorhina tibicen* in more detail, I would recommend greatly increasing the number of specimens and sites included in future

sequencing projects. More specifically, a greater sampling resolution across the arid interior would help resolve questions regarding the phylogeographic history of populations of *G. tibicen* in central Australia, and may additionally be useful when examining the relationship between the distribution of identified subspecies and known genetic clades. Sites where only blood samples were available should also be re-sampled for organ tissue to address the issues of lower coverage and more difficult mitogenome assembly.

## 3.6 Conclusion

In this study, I have reconstructed several whole mitochondrial genomes of *Gymnorhina tibicen*, as well as numerous near-complete mitogenomes, and used them to investigate the phylogeography of the species across continental Australia, Tasmania and Papua New Guinea. I provide data from unsampled regions, including a Western Australian specimen closely related to specimens from Alice Springs and central Victoria, and propose tentative explanations for this pattern. Individuals from Flinders Island in the Bass Strait were closely associated with those from Tasmania. Comparison of the degree of variation among clades for different genes followed similar trends as those observed previously. I also include several recommendations aimed at future studies using whole mitochondrial genomes for phylogeographic study of the Australian magpie.

The mitochondrial genome of *Gymnorhina tibicen* assembled as part of this project is most likely complete. While an earlier study also successfully produced a complete mitogenome, I believe the genome assembled in NOVOplasty has addressed some sources of error acknowledged in the previous study. Additional targeted sequencing of the mitochondrial control region will help further verify the integrity of the assembled genome.

# CHAPTER 4: EFFECT OF CLIMATE ON REPRODUCTIVE SUCCESS IN AUSTRALIAN MAGPIES (*GYMNORHINA TIBICEN*) IN SOUTHERN VICTORIA

## 4.1 Abstract

As climate change continues to influence ecosystem processes, composition, and species resilience, it becomes increasingly important to understand how variation in weather conditions impacts the reproductive success of native wildlife. Long-term ecological datasets are invaluable for these investigations but are difficult to produce for many species. In this chapter, I investigate the relationship between annual variation in climatic variables such as precipitation and temperature, and recruitment in territories of the Australian magpie (*Gymnorhina tibicen*). Data were collected as part of a long-term territory monitoring study covering over 200 territories located in rural Victoria, Australia, over a period of 25 years, including a regional drought from 2001-09. Analysis suggested a positive relationship between recruitment in magpie territories and pre-breeding season rainfall, as well as warmer temperatures during the nesting season. Overall, *G. tibicen* appears relatively robust in the face of variation in local weather, possibly as a result of regional availability of manmade surface water during drought and heatwave conditions.

## 4.2 Introduction

Changing environmental conditions in a region can have implications for many species. As climate changes, a species' ecological niche can be impacted in numerous ways, both abiotic and biotic. Abiotic changes include extreme heat, cold or rainfall events, shifting weather patterns or structural changes to habitat due to changing vegetation. Biotic impacts are those such as changes in timing, composition and abundance of food supplies and the behaviours of local predators. These changes lead to altered demography, having impacts on birth and death rates, distributions and behaviour (McKinnon et al., 2012, Pearce-Higgins et al., 2010). Reproductive success is a demographic trait essential to the continued survival of a population, and many studies have explored the direct and indirect effect of climate variables on recruitment in avian populations (Skagen and Adams, 2012, Gullett et al., 2015, Albright et al., 2011, Brawn et al., 2016).

In temperate bird species, periods of cold, damp weather and increased precipitation have been shown to directly impact productivity by stressing the thermoregulatory capacity of individuals and damaging nests (Skagen and Adams, 2012, Gullett et al., 2015), reducing foraging opportunities and limiting prey activity (Radford et al., 2001). Temperate passerines often experience greater productivity in warmer, drier laying seasons, with increased clutch size (Rotenberry and Wiens, 1991) and larger eggs (Lessells et al., 2002, Stevenson and Bryant, 2000). During incubation, warmer temperatures improve the hatching and fledging success rate (Martin, 1987, Reid et al., 2000), and in the nestling period can lead to increased body mass in chicks and improved immune systems (Ardia et al., 2010), with higher rates of post-fledging survival (Sankamethawee et al., 2009, Grüebler and Naef-Daenzer, 2010).

In contrast, bird populations in arid regions can experience severe drought, leading to drastic reduction in population density (George et al., 1992). Along with low rainfall, abnormally hot periods during the breeding season can lead to lower survival rates of young later in the year, as individuals may invest fewer resources into reproduction during heatwave conditions, producing fewer viable eggs and hatchlings (Albright et al., 2011). Extreme heatwaves, like extreme cold, can overwhelm the physiological limits of bird species and lead to mortality events (Backer et al., 1997), the likes of which have been documented in Australia for almost a century (Finlayson, 1932, Saunders et al., 2011, McKechnie et al., 2012, Chambers et al., 2005a). The impact of these events is most catastrophic when heatwaves and droughts occur simultaneously in the same region, as droughts during relatively cool periods and heatwaves with relatively abundant rainfall are not associated with drastic changes in avian abundance (Albright et al., 2010). There is some physiological basis for this, as individual birds have increased water requirements during periods of high ambient temperature (Williams and Tieleman, 2005).

Environmental changes can lead to shifts in the distribution of a species (Hickling et al., 2006, Parmesan and Yohe, 2003). Most often, climate change leads to changing population densities and poleward range shifts (Virkkala and Lehikoinen, 2014, Lehikoinen et al., 2014) or altitudinal shifts to higher elevations (Pounds et al., 1999, Harrison et al., 2001), as temperatures increase and habitats at higher latitudes/altitudes become more suitable. In Australia specifically, it has been suggested that bird species will need to disperse further and faster than previously estimated to stay in their climatic niche, and not necessarily to higher latitudes (VanDerWal et al., 2012).

Climate change can also have indirect impacts on recruitment in bird populations. Food availability and foraging efficiency are influenced by environmental conditions (Møller et al., 2010), which has downstream effects on the growth rate of chicks and overall abundance (Pearce-Higgins et al., 2010, McKinnon et al., 2012). Many passerines experience high rates of nest predation, which can mask the effects of weather on productivity (Wilson and Arcese, 2006), and the population size and behaviours of predators themselves are also affected by climatic conditions (Adamík and Král, 2008). In seasonal climates, the change in weather conditions may also be associated with phenology, influencing the timing of reproductive events such as breeding, lay-dates, or migration, all of which have a strong impact on reproductive performance (Stevenson and Bryant, 2000, Crick, 2004, Verhulst and Nilsson, 2007).

The response of Australian bird species to climate change and extreme weather events varies among species, with a range of impacts on their distribution, movement, phenology and abundance (Chambers et al., 2005a). As in studies from the northern hemisphere (e.g. Skagen and Adams, 2012), some passerines have exhibited reduced nestling survivorship during periods of increased rainfall (Sandvig et al., 2017). The phenology of some species is impacted by the timing of winter temperatures, as in the superb fairy-wren (*Malurus cyaneus*), which shows an increase in mortality when the timing of moulting coincides with severe minimum temperatures in winter (Cockburn et al., 2008). Increased temperature and reduced rainfall are of particular concern for Australian avifauna, both as long-term trends and localised extreme weather events (Chambers et al., 2005a, McKechnie et al., 2012). Reduced rainfall and higher temperatures, both symptoms of prolonged drought, lead to reductions in canopy foliage, ground litter and shrubs, which leads to reduced invertebrate abundance and therefore less food for insectivorous birds (Bennett et al., 2015), as well as

increased exposure to potential predators (Turcotte and Desrochers, 2003). As many territorial or resident birds do not migrate away from deteriorating conditions (Mac Nally et al., 2009), this presents a danger to many territorial Australian species. Australian passerines generally have low yearly recruitment rates (Yom-Tov et al., 1992), so occasional 'good' years of productivity may not be enough to compensate for breeding failure in dry years, leading to recruitment below replacement levels.

The distribution of the Australian magpie is practically continuous across mainland Australia, with the exception of parts of the arid interior and the northernmost latitudes of the Northern Territory and Queensland (Higgins, 2006, Schodde and Mason, 1999, Marchant et al., 2006). Territories vary greatly in size and capacity depending on their spatial structuring and the quality of foraging habitat available (Hughes and Mather, 1991). In recent years, magpie populations have increased in size due to the proliferation of farmland, which is a preferred habitat (Higgins, 2006). However, studies also suggest that northern Australia represents only marginally suitable habitat for magpies, with lower recorded population densities, less frequent breeding and recorded drops in abundance in more recent years (Gibbs, 2007). It is speculated that the main cause of reduced productivity in 'bad' years is birds choosing not to breed at all, rather than fledgling mortality. Above a certain temperature threshold, foraging effort is significantly reduced, and so birds otherwise capable of reproducing may not have the excess energy to devote to the production of eggs (Edwards et al., 2015a).

A case study by Gibbs (2007) assessed the relationship between climate variation and both timing and 'amount' of breeding in Australian magpies on a broad spatial scale using observations from multiple ornithological groups, using naturally occurring geographic

gradients (altitude and latitude) and the Southern Oscillation Index (SOI) as measures of climate. The principal conclusions were that magpie populations at higher altitudes breed later in the year, the proportion of breeding records increased at higher latitudes, and that years of high SOI correspond to more and earlier breeding. Interestingly, years with high SOI are associated with La Niña events, flooding and high rainfall, which often negatively impacts nesting birds (Sandvig et al., 2017, Skagen and Adams, 2012, Gullett et al., 2015).

The impact of changing weather on reproductive success and recruitment in Australian birds is uncertain, as there are complex relationships between species' biology and climate in Australian birds. There are relatively few studies on the effect of climate change on Australian biota compared to the rest of the world, with many focusing on phenology, particularly the timing of breeding and migration events (Green and Pickering, 2002, Chambers et al., 2005a, Beaumont et al., 2006). Studies support early breeding at higher latitudes and altitudes in many Australian bird species, but no consistent trends associated with year across multiple species, save for significantly earlier breeding in southeast Australia (Gibbs et al., 2011). It has also been highlighted that annual fluctuations in rainfall can have dramatic effects on breeding, even for larger more sedentary seasonal breeders. In a study of bird species in the tablelands of northern New South Wales, of the 46 species monitored, 18 did not breed at all when conditions were unfavourable, and the remaining species, including the Australian magpie, bred significantly less (McLean, 2005).

Due to practical constraints, there are not many long-term (multi-decadal) studies of breeding populations that consider the impact of weather on recruitment, and therefore data availability is limited. This study represents an opportunity to evaluate the impact of weather on

productivity in a widespread and iconic generalist Australian passerine over more than two decades, more than a decade since the last multi-year study on the species (Durrant and Hughes, 2005), using several metrics for breeding success. As climate change emerges as a leading cause for concern for the abundance and health of biota worldwide, information about the relationship between environmental conditions and avian reproductive output will be increasingly relevant for identifying at-risk populations and regions and informing conservation and management efforts.

The Australian magpie (*Gymnorhina tibicen*) makes a suitable study species for several reasons. They are abundant across much of the Australian mainland & Tasmania and are easily identified in the field thanks to their stark coloration and iconic call, allowing for quick and accurate observations. In addition, magpies are a resident, territorial species, generally single-brooded, and breed in a single season each year, which should make it possible to link monthly weather variation to productivity in a local population. Since 1992, a long-term study of an Australian magpie population in rural Victoria has been underway that consists of twice annual observation periods and biological sampling, concluding in 2017. The data and samples from this project have been used in a broad range of population genetic and ecological studies (Baker et al., 2000, Hughes et al., 2001, Toon et al., 2003), but no investigation has yet been made of the relationship between environmental conditions at the study site and annual levels of productivity. Additionally, southeast Australia experienced a prolonged and severe drought from approximately 2001 to 2009 (van Dijk et al., 2013), as did much of the country. This marked change in local weather conditions, combined with the timing of the long-term project, provides an opportunity to investigate the effect that variation in weather has on reproductive success in *Gymnorhina tibicen*. Territory occupancy and demographic data from this population was used for the analysis in this study.

The aim of this study was to investigate the effect of variation in local weather conditions on the productivity of Australian magpie territories. I hypothesized that there would be a significant positive effect on territory productivity from cooler summers, both in the leadup to the breeding season (February-March) and during the nesting and fledging seasons (October/November). It could be reasonably expected that winter months with high rainfall would have a negative impact on the number of fledglings produced later that year (see Sandvig et al. (2017)), but I did not predict a significant negative effect from reduced rainfall. As the study site is mostly pastoral farmland, semi-permanent bodies of water are present year-round in the form of dams and water troughs, and if recruitment is dependent on water availability, the study population might not be as strongly affected by drought as more undeveloped regions. In contrast, since rainfall increases food availability by promoting greater invertebrate abundance, high rainfall before and during the breeding season might indirectly lead to greater reproductive success in breeding magpies.

## 4.3 Materials and Methods

### *4.3.1 Study area and field methods*

Field data was collected over a 23-year period from 1994 to 2016, from a population of Australian magpies (*Gymnorhina tibicen*) distributed on either side of Lambing Gully Road, 5km south of Avenel, Victoria in south-east Australia (36°58'20.8"S, 145°13'37.9"E). Territories were identified and birds were individually banded in 1992 and 1993 before monitoring commenced in 1994, and banding of new birds continued throughout the study. Over the course of the study, a total of 201 different territories were monitored, with individual territories observed for between 1 and 25 years, and individual territories persisting for an average of 9.7 years. Territories were largely adjacent, and were distributed along approximately 12km of road, running from north to south. The sites were mostly located in pastoral farmland, open fields interspersed with sparse tree cover, although many included sections of bush with both understorey and shrub layers.

### *4.3.2 Breeding success parameters*

Territories were surveyed twice a year for 25 years, during two study periods – one in March/April, and one in October/November. As part of a broader study, birds in each territory were banded (with approval from the Australian Bird and Bat Banding Authority) and their occupancy recorded from year to year. As part of each survey, territories were observed over a two week period in March/April and another four week period in Oct/Nov. Membership in a territory required that an individual be observed within the territory bounds on at least three separate watches (20 min territory$^{-1}$) during a study period, similar to other studies of the Australian magpie (Durrant and Hughes, 2005, Hughes et al., 2011). Fledglings are readily distinguishable from adults, as the stark black and white regions of adult plumage

are instead dark grey and light grey in fledglings. The number and identities (where possible) of all occupants of a territory were recorded each visit, including banded and unbanded fledglings and adults. The resulting observations were used to calculate two metrics of reproductive success: for each year, the number of fledglings produced per territory, and the proportion of productive (producing one or more fledglings) territories each year. The mean number of fledglings produced per female in a territory was also considered as a possible metric. However, a Pearson's correlation analysis showed that the mean fledglings per territory and per female in a territory were highly correlated ($r$ = 0.91), so fledglings per female was not retained as a response variable in the analyses.

### *4.3.3 Environmental parameters*

Weather data were obtained from a permanent weather station located 6km north-east of the field sites (Mangalore Airport, 36°53'25.1"S 145°10'54.4"E). Parameters collected included daily observations for minimum, mean and maximum air temperature (°C), wind speed (m $s^{-1}$) and total precipitation (mm). In this analysis, the daily observations for each parameter were converted into monthly averages from January to November. Extreme weather events, such as high wind speeds or rain days above a specific threshold, were not included as they would be reflected in the monthly averages.

Three of the 312 monthly rainfall observations from the selected weather stations were unavailable due to 1-2 weeks of missing observations in those months. After assessing the different methods available for handling missing precipitation data, a multiple linear regression method was used to interpolate the missing values from four neighbouring weather stations (see Appendix table II-A for weather station information). This approach has been

suggested for regions where weather data is available for multiple neighbouring stations at similar altitudes (Sattari et al., 2016).

### *4.3.4 Statistical analyses*

All analyses were conducted in R 3.6.0. First, descriptive statistics (mean, standard deviation and range) were generated for each response and predictor included in the study. Plots of each response variable over the 23 years of the study were generated to help illustrate the overall change in each metric from 1994 to 2016. Response variables were the mean number of fledglings produced per territory each year, and the proportion of territories in the population that were 'productive' (produced one or more fledglings that year). My approach was to model productivity of magpie territories as a function of weather variables and non-climatic variables that may influence productivity (Table 1). All analyses were undertaken at the population level, and weather variables were calculated as monthly means. The packages MuMIn (Barton and Barton, 2019) and glmulti (Calcagno and de Mazancourt, 2010) were used in an information theoretic approach to model comparisons over the predictor set, and the fit of each model was assessed using the Akaike information criterion, corrected for small sample sizes (AICc). General linear models with normal error structure were used for continuous measures (mean number of fledglings produced per territory per year).

First, I investigated the effect of mean monthly temperature, wind speed, and total monthly precipitation on each response variable using linear regression, to check for any obvious effect. Including all variables for all months from January to November would introduce bias into the population-level analyses, due to the small number of observations relative to the number of possible predictors. To address this, a set of 'seasonal' models were tested first.

These models covered weather conditions in the previous summer (Dec-Feb), autumn (Mar-May), winter (Jun-Aug), and Spring (Sep-Nov), leading up to the date of the field observations. Model averaging was used over each 'seasonal' model, in which all possible combinations of predictors were compared, and parameters appearing in the set of 'best' models (i.e. those within 2 AICc points of the most parsimonious model) were included in a year-round model. This approach resulted in my 'year-round' model including mean temperatures in the preceding December, February, March, July, October and November, and rainfall in the preceding December, May and June. Adult population size was included as a non-weather predictor in both yearly models to try to account for population density, as high population density might be expected to have a negative impact on recruitment.

Broad ranges were used because magpies have an extended breeding season – they may breed any time between June and December, but in colder or temperate conditions this is usually restricted to August/September (Kaplan, 2019). Opportunities for field observations were limited to two periods each year, so the exact timing of laying, nesting and hatching events could not be ascertained. It was worth investigating the effect of months leading up to the breeding season, as poor conditions in summer/autumn and harsh winters may determine parental body condition, and therefore ability to invest in reproduction (Robb et al., 2008). Magpies tend to spend about eight months raising the offspring of a single clutch, so despite their extended breeding season, it is unlikely that a second clutch will be raised in a single year unless nest failure occurs very early in the season, and fledglings observed in November should account for most successful recruitment that year.

Table 4.1 Variables used in population-level productivity analyses.

| **Response Variable** | **Climatic Predictors** | **Non-climatic Predictors** |
|---|---|---|
| Mean fledglings per territory | Monthly temp + prec + wind speeds | Adult population size<br>Total pop. size |
| Proportion of territories productive | Monthly temp + prec + wind speeds | Adult population size<br>Total pop. size |

## 4.4 Results

### *4.4.1 Weather variation & productivity outcomes*

Variation in weather conditions and territory productivity for the Seymour population of Australian magpies is described in table 2. Over the 23 years included in this study, magpie populations experienced considerable variation in weather conditions, particularly in spring, with average temperatures ranging from 15.1 to 21.8°C (see Appendix III & V for plots of annual variation by month). Rainfall exhibited considerable variation across all months in the dataset, with some months ranging from as little as <10mm to >100mm across the duration of the study. The proportion of productive territories also varied considerably from year to year, ranging from 38-83%.

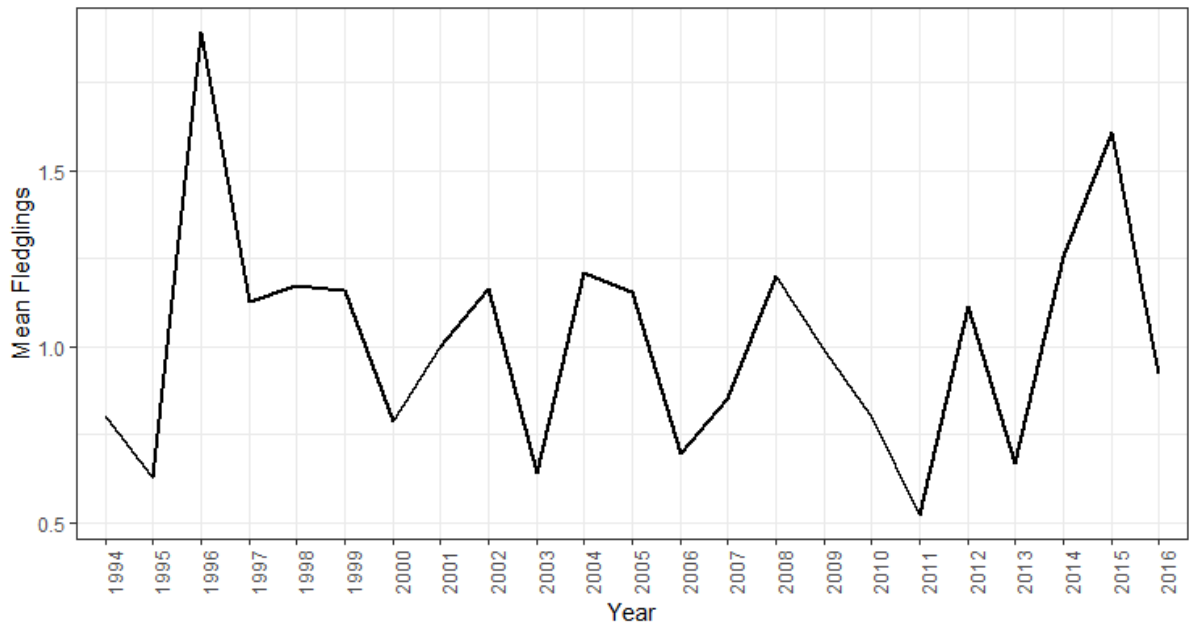


Figure 4.1 Annual mean fledglings produced per territory for the Lambing Gully Road population, from 1994 to 2016.

Table 4.2 Summaries of variation in weather conditions and population-wide productivity outcomes used in productivity analyses. Means, standard deviations and ranges are displayed for each variable from 1994-2016, a period of 23 years, with December of the preceding year.

| **Population-level Responses** | | **Mean** | **SD** | **Range** |
|---|---|---|---|---|
| Mean Fledglings per territory | | 1.02 | 0.32 | 0.52-1.89 |
| % Productive territories | | 0.57 | 0.11 | 0.38-0.83 |
| **Climatic Predictors** | **Month** | **Mean** | **SD** | **Range** |
| Mean Temp | Dec | 20 | 1.5 | 17.2-22.9 |
| | Jan | 22.47 | 1.4 | 19.7-24.5 |
| | Feb | 22.2 | 1.6 | 19.1-24.8 |
| | Mar | 19.1 | 1.2 | 17.2-21.7 |
| | Apr | 14.92 | 1.4 | 12.4-17.2 |
| | May | 11.39 | 0.8 | 10.1-13.4 |
| | Jun | 8.83 | 0.8 | 7.3-10.8 |
| | Jul | 8.01 | 0.7 | 6.7-9.3 |
| | Aug | 8.95 | 0.6 | 7.8-9.8 |
| | Sep | 11.17 | 0.8 | 9.4-12.6 |
| | Oct | 13.98 | 1.4 | 11.2-18.8 |
| | Nov | 17.66 | 1.6 | 15.1-21.8 |
| Precipitation | Dec | 30.9 | 22.9 | 381.2 |
| | Jan | 41.4 | 44.8 | 0.2-172 |
| | Feb | 42.8 | 39 | 1.4-147.8 |
| | Mar | 31.5 | 35.1 | 2.2-133 |
| | Apr | 33.2 | 23.2 | 3.8-81.6 |
| | May | 38.6 | 26.3 | 4.2-95.6 |
| | Jun | 54.9 | 27.9 | 14.6-128.2 |
| | Jul | 53.6 | 26.9 | 12.8-111.2 |
| | Aug | 46.4 | 25.4 | 5-84.2 |
| | Sep | 46.9 | 27.4 | 8.6-105 |
| | Oct | 34.5 | 30.1 | 0-106.4 |
| | Nov | 54.5 | 36.3 | 12.6-150 |
| **Non-Climatic Predictors** | | **Mean** | **SD** | **Range** |
| Adult Population Size | | 263.1 | 83.9 | 155-414 |
| Mean Adults per territory | | 3.314 | 0.67 | 1.91-4.47 |
| Total Population Size | | 439.3 | 85.3 | 317-619 |

*4.4.2 Simple linear regressions*

The simple linear regressions using mean monthly weather data and population-wide recruitment data did not produce many significant relationships (see **Tables 4.3, 4.4 & 4.5**). Mean monthly precipitation and wind speeds showed no significant correlation with the mean fledglings produced per territory. Mean monthly temperature showed a significant correlation in October ($p$ = 0.032), with a 1°C increase in temperature producing a 0.099 increase in the mean fledglings/territory.

Table 4.3 Linear regression summary for mean chicks/territory and monthly temperature.

| **Month** | **Intercept** | **Mean temp.** | **R-squared** | **Model *p*-value** |
|---|---|---|---|---|
| January | 1.784 | -0.034 | 0.021 | 0.512 |
| February | 2.295 | -0.058 | 0.034 | 0.198 |
| March | 2.123 | -0.058 | 0.005 | 0.306 |
| April | 1.395 | -0.025 | 0.011 | 0.626 |
| May | 0.485 | 0.047 | 0.014 | 0.584 |
| June | 0.542 | 0.054 | 0.017 | 0.557 |
| July | 1.779 | -0.095 | <0.001 | 0.327 |
| August | 0.847 | 0.019 | 0.001 | 0.864 |
| September | 0.325 | 0.062 | 0.025 | 0.469 |
| October | -0.37 | 0.099 | 0.2 | **0.032** |
| November | 1.612 | -0.034 | 0.028 | 0.449 |

Table 4.4 Linear regression summary for mean chicks/territory and monthly precipitation.

| Month | Intercept | Mean precip. | R-squared | Model *p*-value |
|---|---|---|---|---|
| January | 1.04 | -0.001 | 0.007 | 0.709 |
| February | 1.058 | -0.001 | 0.014 | 0.587 |
| March | 1.025 | -0.0003 | 0.001 | 0.88 |
| April | 1.019 | -0.00009 | < 0.001 | 0.977 |
| May | 1.166 | -0.004 | 0.057 | 0.141 |
| June | 0.828 | 0.003 | 0.087 | 0.172 |
| July | 1.084 | -0.001 | 0.011 | 0.63 |
| August | 0.983 | 0.001 | 0.003 | 0.804 |
| September | 0.961 | 0.001 | 0.009 | 0.652 |
| October | 1.126 | -0.003 | 0.088 | 0.17 |
| November | 1.048 | -0.001 | 0.004 | 0.761 |

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Table 4.5 Linear regression summary for mean chicks/territory and mean wind speed.

| Month | Intercept | Wind speed | R-squared | Model *p*-value |
|---|---|---|---|---|
| January | 2.356 | -0.074 | 0.069 | 0.205 |
| February | 2.228 | -0.07 | 0.052 | 0.27 |
| March | 1.131 | -0.007 | 0.001 | 0.888 |
| April | 1.127 | -0.008 | 0.001 | 0.889 |
| May | 1.558 | -0.046 | 0.037 | 0.355 |
| June | 1.267 | -0.02 | 0.007 | 0.698 |
| July | 1.822 | -0.067 | 0.033 | 0.19 |
| August | 0.779 | 0.019 | 0.007 | 0.682 |
| September | -0.082 | 0.081 | 0.109 | 0.107 |
| October | 1.106 | -0.006 | < 0.001 | 0.926 |
| November | -0.167 | 0.072 | 0.079 | 0.173 |

*4.4.3 General linear models*

In my analysis of population-wide breeding success (Table 4.6), there was no effect of weather during the preceding summer, early autumn or late winter on territory productivity. Precipitation during May was suggested to have a negative impact on the mean number of fledglings produced, but this was not significant and possessed little explanatory power compared to the effect of rainfall during June (partial $R^2$=0.24), where a 1mm increase in rainfall produced a 0.005 increase in the mean fledglings produced per territory. Temperature in October also had a significant positive effect (partial $R^2$=0.36), with a 1°C increase producing a 0.134 increase in the mean fledglings produced per territory that year. Temperature in November had a slight negative effect, but this was also not significant and had low explanatory power.

Table 4.6 The effects of weather on population-wide productivity in the Seymour population of Australian magpies from 1994-2016. The mean number of fledglings produced by each territory annually was used as the metric of productivity.

| Variable | Estimate | SE | Partial $R^2$ | *sig.* |
|---|---|---|---|---|
| **(a) Fledglings per territory: $R^2$ = 0.44, 3 models** | | | | |
| *Intercept* | -0.893 | 0.78188 | - | |
| May rainfall | -0.003 | 0.00226 | 0.08 | 0.67 |
| June rainfall | 0.005 | 0.00229 | 0.239 | **0.02** |
| October temp | 0.134 | 0.04399 | 0.361 | **0.002** |
| November temp | -0.051 | 0.04223 | 0.079 | 0.67 |

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## 4.5 Discussion

In this chapter, I have used 23 years of monitoring data from 201 territories of Australian magpie to investigate the effect of short-term annual variation in weather on productivity outcomes. During this time period, the study region experienced a prolonged drought (from 1996 to 2010, see BOM (2015)), and it was anticipated that this would have an impact on productivity. Additionally, temperature in the study region varied considerably over the duration, particularly in summer, and a slight upward trend in mean monthly temperature can be observed across the duration of the study (see supplementary table III). This variation in weather has had a significant effect on the metrics of breeding success used in this study.

Weather in most months leading up to the observation periods did not influence the number of fledglings produced per established territory. However, there was a strong positive relationship between the mean number of fledglings per territory and precipitation in June, which varied from 14.6mm to 128.2mm and peaked in 2010. Rainfall in early winter has been observed to increase rates of nest destruction and fledgling mortality in some species (Skagen and Adams, 2012, Sandvig et al., 2017) and has been reported as having a negative impact on breeding success in several larger Australian bird species (Rae, 2017, Olsen and Olsen, 1989). In Australia, variation in rainfall is particularly important in determining the abundance of insect populations (Drake, 1994). As Australian magpies are predominantly insectivorous, it is plausible that increased precipitation in winter leads to an increase in the abundance of prey in later months, improving the health of breeding females and subsequently improving their breeding success rate (Møller et al., 2010), outweighing any losses due to increased rainfall. A similar positive response has been observed in Superb fairy-wrens (Cockburn et al., 2008), which was also speculated to be a result of increased food availability.

October temperatures also had a strong positive effect on the number of fledglings produced per territory, ranging from 11.2-18.8°C and peaking in 2015 (see Table 4.2, supplementary figure III). This association could arise from better environmental conditions for foraging and thermoregulation during the post-fledging period, which has been noted to be a critical period for juvenile mortality in passerine species (Naef-Daenzer et al., 2001, Sankamethawee et al., 2009). Warmer weather during the nesting period has been linked to increased fledgling body mass and survival rates in tree swallows (Ardia et al., 2010) and improved post-fledgling survival in barn swallows (Grüebler and Naef-Daenzer, 2010) and long-tailed tits (Gullett et al., 2015).

Australian magpies increase in abundance with access to artificial water sources, which may help them resist the negative effect of drought, particularly in heavily transformed pastoral landscapes with numerous dams and cattle troughs (Reid and Fleming, 1992). Australian magpies could be expected to tolerate the change better than smaller passerines, as heat tolerance has been observed to increase with body mass (McKechnie et al., 2017). However, increases in minimum and mean temperatures during the nesting season and frequency of drought events in the leadup to nesting would lead to increased nestling and fledgling mortality, with populations reducing in size and offspring dispersing further to find suitable habitat.

There are additional life history traits that could be considered when evaluating breeding success and recruitment. The loss of eggs in each clutch, as well as the loss of chicks and fledglings, could be included whether it is a result of attrition from poor environmental conditions or predation. Many studies of passerine reproductive success monitor clutches laid

by individual females (Gullett et al., 2015, Norris et al., 2004, Weatherhead, 2005, Kitaysky and Golubova, 2000), but this is difficult for Australian magpies due to the relative inaccessibility of their nests, their extended breeding season, and reported instances of females laying their eggs in the nests of other females (Kaplan, 2019, Hughes et al., 2003). *Gymnorhina tibicen* is also documented as having widely varied rates of extra-pair paternity (EPP) across its range, sometimes as high as 82% (Durrant and Hughes, 2005), which complicates measures of per territory reproductive success. Inter-annual variation in rates of EPP may also be influenced by variation in local weather conditions. In less favourable conditions such as extreme cold or drought, less energy may be available with which to pursue extra-pair activities, as in the snow bunting (*Plectrophenac nivalis*) (Hoset et al., 2014). Additionally, the phenology of magpies is known to be affected by variation in weather (Gibbs, 2007, Rollinson and Jones, 2002, McLean, 2005), and more precise estimates of weather conditions leading up to and during individual nesting attempts would aid in assessment of the relationship between recruitment and weather.

Data regarding the overall mortality and reproductive rate of magpies is scarce. Early studies by Carrick (1972) suggest that for every seven offspring that successfully hatch, only one survives to adulthood. This estimate worsens considerably where quality of the territory is diminished, with 'peripheral' territories that offer less shelter having as low as 1 in 39 hatchlings survive to adulthood. The low annual rates of recruitment in my study lend support to the idea that the overall viability of a population depends on the longevity of individuals that reach adulthood and produce several offspring of their own (Gullett et al., 2015, Pearce-Higgins et al., 2010).

## 4.6 Conclusion

In this chapter, I have used data collected from a population of Australian magpies in central Victoria to make inferences about the impact of variations in weather on the reproductive success on territorial units of birds. The analysis I performed suggests that increased rainfall in June and warmer temperature in October both lead to increases in the number of fledglings per territory. This could be linked to an increase in invertebrate abundance and food availability through winter and reduced thermoregulatory stress on chicks and fledglings due to earlier summers. As droughts are expected to increase in frequency and severity over the next several decades, and temperatures are expected to increase, these factors may result in reduced foraging efficiency, reduced water availability and increased thermoregulatory stress for passerines such as magpies. Future studies could record reproductive success of female magpies at the individual level and take into account the size and quality of habitat within each territory when assessing the impact of environmental conditions on recruitment.

# CHAPTER 5: GENERAL DISCUSSION

### *5.1.1 Summary of Findings*

The projects in this thesis represent several advancements in our understanding of *Gymnorhina tibicen*, particularly the genetic relationships among populations across its distribution, and the resilience of local populations to fluctuations in environmental conditions.

In Chapter 2, I have successfully developed a reduced-representation genomic dataset for *G. tibicen* and characterised the genomic diversity of ten populations distributed across the Australian mainland and Tasmania. Broad trends in genetic structure and demographic history were in line with my initial expectations. Though the timing of the demographic changes was slightly earlier than anticipated, the trends themselves supported my initial hypotheses – that of a constant population expansion in eastern populations of *Gymnorhina tibicen,* and in the western group, a historical expansion and contraction followed by stable population size approaching the present, as suggested in Toon et al. (2007). It is also possible that, as the sites included in the analysis are relatively close together (compared to the true geographic extent of the clusters), the sets of individuals used share a most recent common ancestor earlier than expected.

Extended Bayesian Skyline analyses are well suited for investigating demographic changes over hundreds of thousands, to millions of years (Heled and Drummond, 2008, Dorrington et al., 2020, Barnett et al., 2014), but the relatively short timescale and recency of coalescent events in the *Gymnorhina tibicen* populations investigated here may make inferring historical

population size more difficult. A more precise method of calculating the molecular clock rate may also be needed than that used here, which was originally used to reconstruct demographic history over a period of almost 1 million years (Lado et al., 2020). As covered in 2.6, there are also several areas that could be readily improved upon to further investigate the demographic history of the Australian magpie. To better characterize genomic diversity across the species' entire distribution, it would be appropriate to sample a larger number of individuals distributed more evenly across *G. tibicen*'s range, rather than focusing on multiple individuals from each of a small number of sites. This would help mitigate some of the bias that can be introduced into coalescent analyses such as the Extended Bayesian Skyline Plot due to limited sampling design (Heller et al., 2013), and ensure that the genetic diversity within each region is better represented in the analyses.

The RADseq dataset generated in this project will be able to be combined with data generated in future reduced representation sequencing projects, provided the same restriction enzymes and analogous sequencing platforms are used. As the avian genome is highly conserved (Zhang et al., 2014), and small in comparison to many other taxa (Ellegren, 2013), genomic studies of birds are likely to remain an attractive prospect in future so long as the cost of sequencing remains proportionate to the size of the genome in question. The genomic diversity of *Gymnorhina tibicen* across much of its distribution is yet to be characterized, and while the species is not currently of conservation concern, there are still questions to be investigated regarding the influence of cyclic aridification during glacial maxima on the species' distribution and demographic history, and the maintenance of several plumage morphs in the face of gene flow (Dobson et al., 2012, Prince, 2015).

In Chapter 3, I have assembled complete mitochondrial genomes of *G. tibicen*, and subsequently used them to explore facets of the species' phylogeography. Prior to this project, one attempt had been made to assemble a complete mitogenome prior to this project (Grealy et al., 2019), which produced a near-complete sequence with the exception of a short repetitive sequence situated in the D-loop. I hoped to build upon their success by using alternative tissue types, sequencing and assembly methods, using software with built-in methods of handling short repetitive sequences (Dierckxsens et al., 2017). Five whole mitogenomes were assembled that I believe do not possess the same gap as the first published, and I have used mitochondrial sequence data from both the complete assemblies and partial mitogenomes to examine the phylogenetic relationships between samples.

The extension of what has previously been identified as a predominantly 'eastern' cluster to Lake Carnegie in WA is notable. The arid interior is difficult to sample as thoroughly as the more temperate coastlines of the Australian continent, due to relatively low population densities for many taxa as well as practical constraints (Piccolo et al., 2020, Lindenmayer et al., 2012). It is therefore more difficult to accurately capture the patterns of genetic structure and diversity that exist across the centre of the continent. There remains the possibility that the patterns observed here reflect relatively recent (post-European colonization) dispersal from eastern populations, which has been raised in prior studies of *G. tibicen* (Toon et al., 2007) as an explanation for the closer relationship of Alice Springs specimens to eastern populations. Some species whose distributions are restricted by proximity to streams or rivers can disperse further into arid regions thanks to the provision of manmade water sources, a consequence of expanding pastoralism (Saunders and Curry, 1990, Reid and Fleming, 1992, Letnic, 2007). Furthermore, phylogeographic patterns in other Australian bird species have suggested broadly similar patterns of dispersal inland from eastern populations, while

populations on the west or southwestern coast tend to remain more highly structured (McLean et al., 2017). In the case of *G. tibicen*, this has previously been attributed to more philopatric behaviour in western populations (Baker et al., 2000). In my discussion of these findings (Chapter 3.5), I suggest two main improvements for further mitogenomic studies of *G. tibicen:* sampling at a higher resolution across the distribution of the Australian magpie, particularly across gaps such as the arid interior, the Kimberley region of western Australia, and northern Queensland, and the selection of organ tissue other than blood for DNA extraction to try and mitigate some of the obstacles encountered during assembly.

My final project (Chapter 4) involved the analysis of long-term recruitment data of a group of territories situated in rural Victoria. This project was borne of the opportunity to contribute to a series of long-term studies conducted at the site by Prof. Jane Hughes. For my part, I have examined the relationship between variation in local weather conditions and reproductive success in territories of *G. tibicen* using 25 years of recruitment data covering over 200 territories. My principal findings were that the Australian magpie is relatively resilient in the face of drought and other adverse conditions, perhaps due to the flexibility of its diet and the abundance of artificial groundwater in the region, and exhibits higher levels of recruitment during years where winter precipitation is higher. I tentatively attribute this to increased invertebrate abundance and its positive effect on the health of breeding individuals, as in other bird species (Møller et al., 2010, Cockburn et al., 2008).

Climate projections do not bode well for most Australian fauna. Continued increase in mean temperatures, decreased cool season rainfall and greater intensity of short duration extreme rainfall events and high intensity storms, and longer fire seasons in southern and eastern

Australia, have all been predicted and are starting to be observed across the continent (Bureau of Meteorology and CSIRO, 2020). In addition, long-term trends are beginning to emerge across many taxa that could be a consequence of these changes (Hughes, 2003, Chambers et al., 2005b, Byrne et al., 2008, Gibbs et al., 2011, McKechnie et al., 2012), though shifts in biodiversity can be difficult to attribute to a singular cause. Some changes, such as shifts in distribution further south, to higher latitudes, or along changing rainfall contours, are more likely to be a result of climate change as opposed to anthropogenic impact (Hughes, 2003).

In the case of *Gymnorhina tibicen*, I suggest that the species is in a relatively good position to endure worsening climate conditions in the coming decades. Changing fire regimes, land clearing and grazing all compound the impacts of climate change on the landscape and on native species, but *G. tibicen* is known to occupy a broad range of habitat types ranging from intact eucalypt woodland to heavily grazed lawns and pastures (Martin and Green, 2002, Higgins, 2006). Furthermore, as mentioned in sections 3.2 and 4.2, the species has a propensity for open farmland, a generalist diet, and cohabitates relatively easily with humans (despite its reputation for aggressive, territorial behaviour) (Higgins, 2006, Kaplan, 2019). This suggests that despite the impact of reduced rainfall and higher mean temperatures on reproductive effort (McLean, 2005, Edwards et al., 2015a), the Australian magpie may be more resilient than other native species, as it is able to readily access artificial surface water across much of its habitat thanks to the spread of pastoralism and other human development (Davies, 1977, Letnic, 2007, Reid and Fleming, 1992).

*5.1.2 Synthesis of Findings*

The creation of genomic datasets for use in studies of non-model organisms has become increasingly affordable and efficient over the decades (Hudson, 2008, McCormack et al., 2013, Luikart et al., 2003). For birds in particular, genomic techniques have permitted insight into the impact of environmental factors on avian DNA (Avise and Walker, 1998, Edwards et al., 2015b), the reconstruction of evolutionary histories both within and among related species (McCormack et al., 2012), , the identification of cryptic genetic variation (Weir et al., 2016)) and allowed us to better inform conservation efforts by delineating population structure and assessing the genetic diversity of populations in greater depth than ever before (Toews et al., 2018, Ewart et al., 2020, Funk et al., 2012, Toews et al., 2016).

As these next-generation sequencing approaches become more readily available, it becomes more feasible to treat individual genomic techniques as part of a broader 'toolbox' we can use to answer evolutionary and ecological questions in conjunction with other forms of data. In Chapters 2 and 3, I have used data from nuclear and mitochondrial genomes to make inferences regarding the phylogeography, population genetic structure and diversity of populations of *Gymnorhina tibicen.* Generational changes in recruitment of a population as a result of changing environmental condition, such as those investigated in Chapter 4, could be considered a microcosm of the same processes that drive changes in population size over geological timescales, as I have done using RADseq data in Chapter 2. Collectively, these projects contribute to our understanding of the ecology and evolutionary history of *Gymnorhina tibicen*, and can be expanded upon in future studies of the species.

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# APPENDIX

***Appendix I.*** Sample IDs and locations for all *Gymnorhina tibicen* and *Cracticus nigrogularis* specimens used in this study. Samples marked by an asterisk (*) were provided by the Australian National Wildlife Collection, all others were obtained from the Molecular Ecology laboratory collection at Griffith University.

| Number | ID | Name | State | Latitude | Longitude | Tissue Type |
|---|---|---|---|---|---|---|
| NM1 | Esp | Esperance | WA | -33.85 | 121.883 | Blood |
| NM2 | Alb | Albany | WA | -35.029 | 117.891 | Blood |
| NM3 | Pth | Perth | WA | -31.96 | 115.833 | Blood |
| NM4 | Plb | Pilbara (Geraldton) | WA | -27.706 | 114.176 | Blood |
| NM5* | Ynr | Yannanarie | WA | -22.8658 | 114.9492 | Liver |
| NM6* | LkC | Lake Carnegie | WA | -25.9103 | 122.3561 | Liver |
| NM7 | KimA | Kimberley A | WA | -16.821 | 124.916 | Blood |
| NM8 | KimB | Kimberley B | WA | -15.704 | 126.381 | Blood |
| - | Mh | Busselton | WA | -33.65 | 115.351 | Blood |
| - | Bn | Mandurah | WA | -32.517 | 115.768 | Blood |
| NM9 | Mat | Mataranka | NT | -14.92 | 133.069 | Blood |
| NM10 | Kna | Kiana | NT | -17.243 | 136.183 | Blood |
| NM11 | Als | Alice Springs | NT | -24.054 | 132.732 | Blood |
| NM12 | Cht | Charters Towers | QLD | -20.04 | 146.15 | Blood |
| NM13 | Rck | Rockhampton | QLD | -23.22 | 150.3 | Blood |
| NM14 | Mry | Maryborough | QLD | -25.532 | 152.682 | Blood |
| NM15 | Brs | Brisbane | QLD | -27.587 | 152.86 | Blood |
| NM16 | Grf | Grafton | NSW | -29.676 | 152.927 | Blood |
| NM17 | Ora | Orange | NSW | -33.299 | 149.07 | Blood |
| NM18 | Glb | Goulburn | NSW | -34.757 | 149.727 | Blood |
| NM19 | Row | Rowsley | VIC | -37.728 | 144.386 | Blood |
| NM20 | Ouy | Ouyen | VIC | -35.071 | 142.31 | Blood |
| NM21 | Phi | Phillip Island | VIC | -38.473 | 145.238 | Blood |
| NM22* | Ade | Adelaide | SA | -34.6481 | 137.7536 | Liver |
| NM23 | Ced | Ceduna | SA | -32.07 | 133.4 | Blood |
| NM24* | Fli | Flinders Island | TAS | -40.013 | 148.087 | Liver |
| NM25 | Lnc | Launceston | TAS | -41.437 | 147.136 | Blood |
| NM26 | Hob | Hobart | TAS | -42.867 | 147.322 | Blood |
| NM27* | PNG | Papua New Guinea | PNG | -8.85649 | 141.25822 | Liver |
| NM-PB1 | PB1 | Pied Butcherbird 1 | - | - | - | Blood |
| NM-PB2 | PB2 | Pied Butcherbird 2 | - | - | - | Blood |

***Appendix II.*** Interpolation of missing rainfall data

For my investigation into the effect of weather variables on the breeding success of the Australian magpie (*Gymnorhina tibicen*), I have used observations from a permanent weather station located at the Mangalore airport, 6km from the north end of the field sites (36°53'25.1"S 145°10'54.4"E). Monthly observations of minimum and maximum temperatures, mean temperatures, wind speeds and rainfall were available for the study period (1991-2016), but three months had missing rainfall data due to a lack of observations for 1-2 weeks during those months. After reviewing half a dozen papers suggesting different approaches for interpolating the missing data, I've chosen to follow the recommendation from Sattari et al. (2016), which suggests that for semi-arid regions where weather data is available from neighbouring stations at similar altitudes, a multiple linear regression approach is sufficient.

To use this approach, I required data from neighbouring weather stations. I selected four additional stations at distances between 8-28km from my study site and at comparable elevations. Using a multiple linear regression on the total monthly precipitation data for all months between Jan 1991 and Dec 2016, I interpolated precipitation measurements for the three missing values. It should be noted that while the Avenel weather station (BOM station ID 88002) is a shorter distance from the study site, weather data was not available for many years of the study and so the Mangalore station data (ID 88109), which was almost complete, was used for the analysis instead.

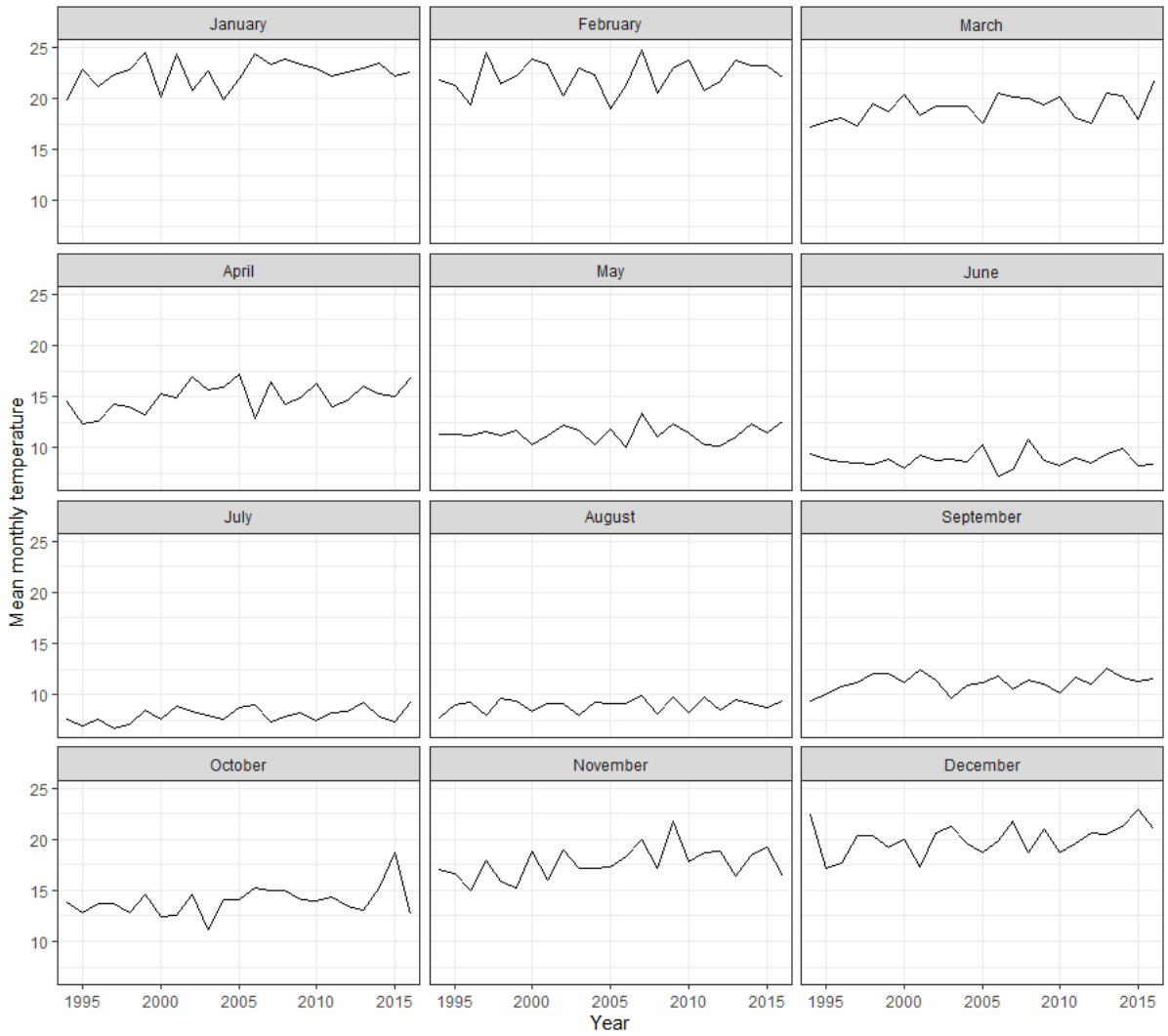
**Appendix II-A.** Lat/Longs, elevation and distance to the study sites for the Mangalore Airport weather monitoring station (in italics) and the four other stations used to interpolate missing rainfall data.

| | Location Name | Latitude | Longitude | Elevation | |
|---|---|---|---|---|---|
| | Study Site Centre | -36.97 | 145.23 | Variable | |
| **BOM Station ID** | **Station Name** | **Latitude** | **Longitude** | **Elevation (m)** | **Distance (km)** |
| 88002 | Avenel Post Office | -36.89 | 145.23 | 152 | 8.89 |
| *88109* | *Mangalore Airport* | *-36.89* | *145.19* | *141* | *9.58* |
| 88053 | Seymour Shire | -37.03 | 145.15 | 145 | 9.75 |
| 88124 | Tabilk Winery | -36.83 | 145.09 | 131 | 19.93 |
| 81019 | Nagambie | -36.72 | 145.17 | 122 | 28.31 |

The resulting formula is as follows:

$$Mng.rain = -1.53013 + 0.46822 * Ave.rain + 0.08709 * Sey.rain + 0.08270 * Nag.rain + 0.34886 * Tab.rain$$

***Appendix III.*** Mean monthly temperature for the Mangalore airport weather station, from 1994 to 2016.

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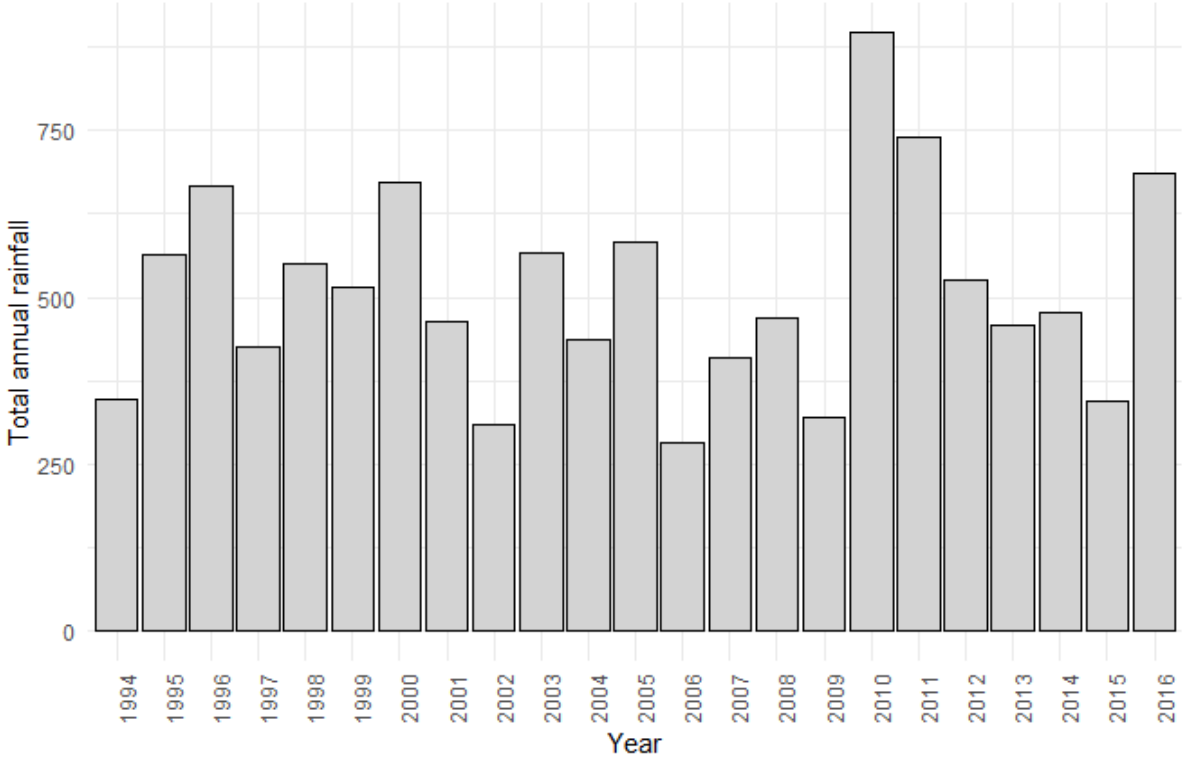
***Appendix IV.*** Total annual precipitation recorded at the Mangalore airport weather station, from 1994 to 2016.

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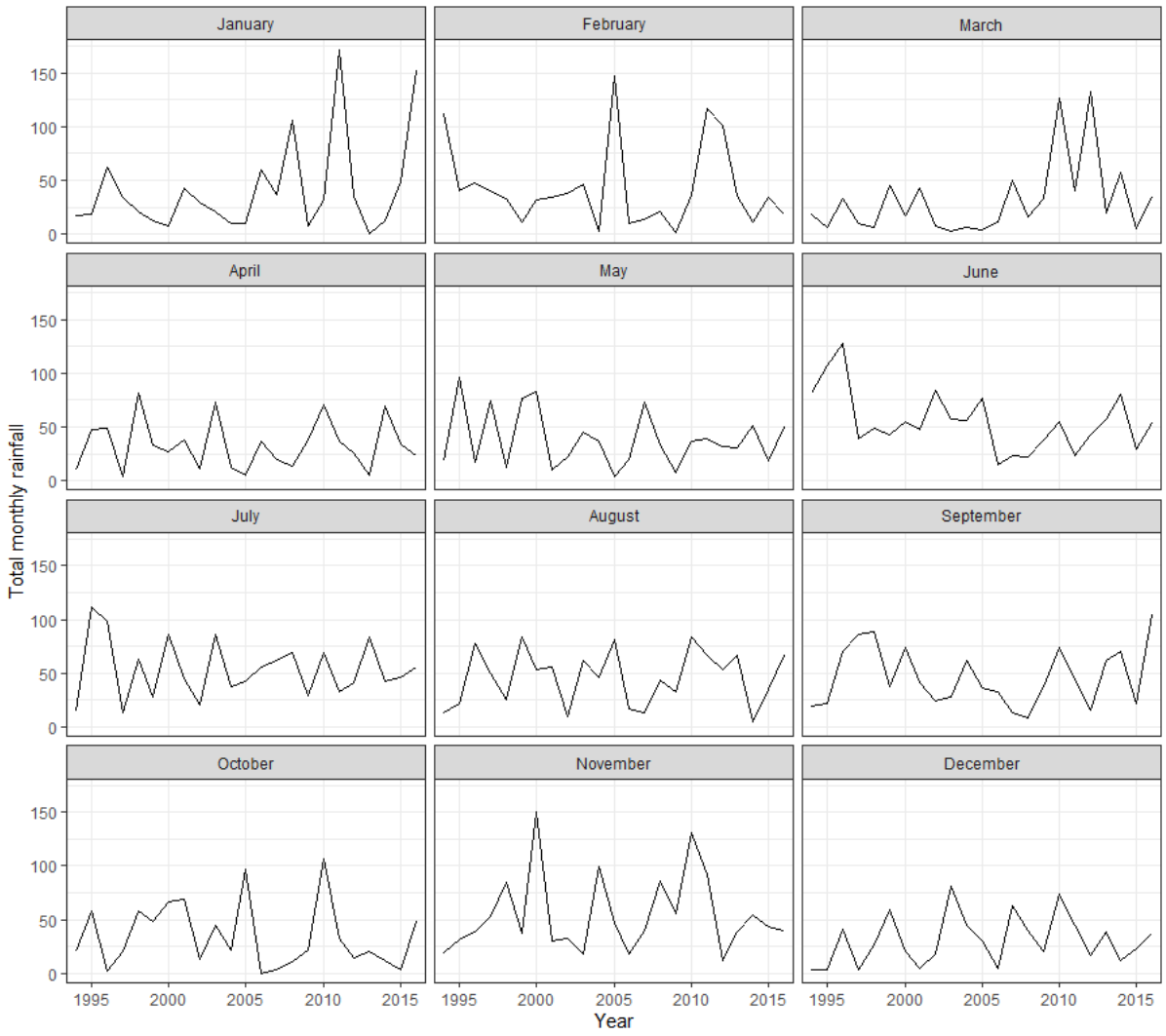
***Appendix V.*** Total monthly rainfall for the Mangalore airport weather station, from 1994 to 2016.

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***Appendix VI.*** Simple linear regression results for mean monthly temperatures, rainfall and wind speeds, using the mean fledglings per territory as the response variable.

| Month | Intercept | Mean temp. | R-squared | Model *p*-value |
|---|---|---|---|---|
| January | 1.784 | -0.034 | 0.021 | 0.512 |
| February | 2.295 | -0.058 | 0.034 | 0.198 |
| March | 2.123 | -0.058 | 0.005 | 0.306 |
| April | 1.395 | -0.025 | 0.011 | 0.626 |
| May | 0.485 | 0.047 | 0.014 | 0.584 |
| June | 0.542 | 0.054 | 0.017 | 0.557 |
| July | 1.779 | -0.095 | <0.001 | 0.327 |
| August | 0.847 | 0.019 | 0.001 | 0.864 |
| September | 0.325 | 0.062 | 0.025 | 0.469 |
| October | -0.37 | 0.099 | 0.2 | **0.032** |
| November | 1.612 | -0.034 | 0.028 | 0.449 |

| Month | Intercept | Mean precip. | R-squared | Model *p*-value |
|---|---|---|---|---|
| January | 1.04 | -0.001 | 0.007 | 0.709 |
| February | 1.058 | -0.001 | 0.014 | 0.587 |
| March | 1.025 | -0.0003 | 0.001 | 0.88 |
| April | 1.019 | -0.00009 | < 0.001 | 0.977 |
| May | 1.166 | -0.004 | 0.057 | 0.141 |
| June | 0.828 | 0.003 | 0.087 | 0.172 |
| July | 1.084 | -0.001 | 0.011 | 0.63 |
| August | 0.983 | 0.001 | 0.003 | 0.804 |
| September | 0.961 | 0.001 | 0.009 | 0.652 |
| October | 1.126 | -0.003 | 0.088 | 0.17 |
| November | 1.048 | -0.001 | 0.004 | 0.761 |

| Month | Intercept | Wind speed | R-squared | Model *p*-value |
|---|---|---|---|---|
| January | 2.356 | -0.074 | 0.069 | 0.205 |
| February | 2.228 | -0.07 | 0.052 | 0.27 |
| March | 1.131 | -0.007 | 0.001 | 0.888 |
| April | 1.127 | -0.008 | 0.001 | 0.889 |
| May | 1.558 | -0.046 | 0.037 | 0.355 |
| June | 1.267 | -0.02 | 0.007 | 0.698 |
| July | 1.822 | -0.067 | 0.033 | 0.19 |
| August | 0.779 | 0.019 | 0.007 | 0.682 |
| September | -0.082 | 0.081 | 0.109 | 0.107 |
| October | 1.106 | -0.006 | < 0.001 | 0.926 |
| November | -0.167 | 0.072 | 0.079 | 0.173 |

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