The ecology of gene flow between two subspecies of thick-billed grasswren (*Amytornis modestus*)

by

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Cover image: Sketch of thick-billed grasswren (*Amytornis modestus*) on top of a chenopod shrub by Paul Vagnarelli.

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Thesis summary

For decades, population divergence associated with speciation was thought to require the absence of gene flow. Recent empirical studies challenge this view by showing that divergence in the presence of gene flow is common. This study investigates interactions between gene flow and divergence at a landscape scale in two thick-billed grasswren subspecies (*Amytornis modestus indulkanna* and *A. m. raglessi*) in arid South Australia. This thesis tests how gene flow between subspecies affects or is affected by the distribution of different markers of divergence. These included (1) a mitochondrial gene, (2) morphology, (3) ecology, (4) nuclear genes, and (5) behaviour. For the first time, I detected thick-billed grasswrens within an area between that previously thought to be the subspecies' core distributions. Although there were divergent morphotypes and mitochondrial haplotypes associated with each subspecies core area, the intervening area and the core area for *A. m. raglessi* contained both mitochondrial haplotypes and the intervening area had intermediate morphotypes. I conclude that the subspecies have a parapatric distribution and are also interbreeding. The distribution of different vegetation types correlated with areas of high and low gene flow. Each subspecies' core area (low gene flow) had different vegetation types and the region of parapatry (high gene flow) had a mixture of both subspecies-specific vegetation types suggesting that natural selection may prevent high gene flow in the core distribution areas. Thick-billed grasswrens were absent from the vegetation type found on sand dunes, which may be a landmark that demarcates where allopatric divergence between the subspecies occurred in the past. Patterns of gene flow across the landscape revealed that gene flow was asymmetric from *A. m. indulkanna* to *A. m. raglessi.* Using recorded song from both subspecies, I measured the territorial response of each subspecies to hetero- and con-subspecific song. *A. m. indulkanna* responded more frequently to hetero-subspecific song

compared to *A. m. raglessi* suggesting that stronger territorial behaviour in *A. m. indulkanna* may be a mechanism for asymmetric gene flow. This thesis answers questions about the pattern of gene flow at a landscape scale in a contemporary threatened songbird species. Ongoing gene flow between subspecies indicates that subspecies are an appropriate classification for these populations. Given the finding that vegetation type creates stepping zones for high gene flow, the thesis reveals the role of habitat connectivity for increasing adaptive potential and persistence in a region of parapatry. The discovery that asymmetric territorial response to intruder subspecies correlates with the direction of gene flow shows that behaviour is an important factor for introgression in an extant bird species. Finally, these patterns of gene flow and divergence at a landscape scale show how evolutionary studies can be used to manage Australia's endemic biodiversity.

Declaration

'I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.'

Amy Lee Slender

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Statement of Authorship/Contribution

Chapters 1 & 6: ALS

Chapter 2:

Data collection: ALS, ML

Laboratory analysis of DNA samples: ALS

Statistical analyses: ALS

Manuscript writing and editing: ALS, MGG, SK, ML

Chapter 3:

Data collection: ALS, ML

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Chapter 4:

Data collection: ALS, ML

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All research procedures reported in this thesis follow the guidelines for the use of animals in research (Flinders University) and were approved by the Animal Welfare Committee of Flinders University (permit: E385) and the Government of South Australia (permit: Z24699). The banding of birds was approved by the Australian Bird and Bat Banding Scheme (ABBBS authority: 2601 and 3108).

Publications associated with this thesis

Information from this thesis has been published in peer reviewed journals as followed:

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Chapter 1 General introduction

Australia harbours a rich natural history that provides a lens through which to examine fundamental evolutionary processes. The evolution and diversification of songbirds in Australia is a hallmark of its natural heritage, yet much remains to be understood about the mechanisms that shape population divergence and ultimately speciation. Within the Maluridae, fairywrens in the genus *Malurus* are one of the most studied groups in Australia (Buchanan and Cockburn 2013), while, aside from phylogeographic studies, grasswrens in the genus *Amytornis* are relatively unstudied (Joseph *et al.* 2013). Grasswrens occur in semi-arid Australia in country that has been impacted by grazing and aridification. Perhaps for this reason many grasswrens are threatened with extinction (Louter 2016). This thesis addresses mechanisms of population divergence in a common and threatened grasswren subspecies in semi-arid Australia with the view to understanding ecological and behavioural parameters associated with gene flow.

Evolutionary processes

Biodiversity changes over time through species extinctions and evolution (Coyne and Orr 2004; Raup 1994). According to the biological species concept, speciation occurs when two genetic lineages diverge to an extent that they become reproductively isolated regardless of whether there is a geographic barrier to gene flow or not (Cook 1906; Smadja and Butlin 2011). Genetic divergence and introgression affect the balance in genetic variation within and between populations, which has implications for the probability of a speciation event to occur. Divergence causes genetic variance within related populations to decrease and reduces the overlapping genetic variation. In contrast, introgression results in greater overlapping

genetic variation. There are three evolutionary processes that control the balance between divergence and introgression (Slatkin 1977; Slatkin 1987). Two processes, genetic drift and natural selection, are dependent on the occurrence of novel genotypes or phenotypes that facilitate population divergence. The third process, gene flow, is dependent on dispersal and hybridisation and results in increased introgression (Slatkin 1987). These processes are not exclusive and they interact with each other either to reinforce or diminish the likelihood of speciation.

Population divergence can be shaped through genetic drift, an evolutionary process whereby allelic frequencies change randomly over time. Genetic drift leads to divergence when different populations recruit different alleles or the frequency of a single allele changes so that it becomes more common in one population and less common in another. This can occur through assortative mating, isolation by distance, or when populations are geographically isolated (Dieckmann and Doebeli 1999; van Strien *et al.* 2015). Alleles affected by genetic drift are likely to have a neutral effect on survival and mate choice because changes in allele frequencies are random. Genetic drift is more likely to cause population divergence when there is low gene flow and weak natural selection (Wright 1931); it is an important factor that decreases genetic variation within isolated populations and reduces the overlapping genetic variation between isolated populations.

Natural selection is perhaps the most widely regarded evolutionary process given its revolutionary articulation by Darwin (1859), and is now known to occur via non-random changes in allele frequencies as particular traits are favoured or selected against. Unlike genetic drift, divergence through natural selection can be dependent on variation in sexual preference or environmental niche. This variation may or may not be associated with different geographic locations (Dieckmann and Doebeli 1999). Natural selection increases the

frequency of particular alleles in a particular population as those alleles improve survival or increase sexual success in those individuals (Fisher 1930; Lande 1982). Populations that contain individuals that prefer different sexual signals or that occur in different environments will diverge because different genotypes and phenotypes increase in frequency (Benkman and Mezquida 2015; Boag and Grant 1981; Mayr 1963; Schluter and Grant 1984a). Genetic drift can reverse the effects of natural selection when natural selection affects alleles that occur at low frequencies (Endler 1977). This is because low frequency alleles are less likely to be inherited in the following generation. Gene flow can also reverse the effects of natural selection provided that natural selection has only a weak survival or sexual advantage (Coyne and Orr 2004; Endler 1977).

Sexual selection is a form of natural selection that specifically describes variation in reproductive fitness including selection for traits that confer reproductive benefits (e.g. mate attraction, offspring production). When sexual selection is in force, individuals with traits that confer reproductive benefits will sire more offspring. Sexual fitness differs according to variation in behavioural, physical or physiological traits that affect an individual's chance of breeding (reviewed in Andersson and Iwasa 1996). Sexually selected traits allow an individual to outcompete same sex rivals or increase their appeal to a mate or more mates (Grant and Grant 2008) and can also operate at the level of extra-pair copulation (Birkhead and Moller 1992; Rowe and Pruett-Jones 2013). Sexual selection is a process that increases or maintains genetic variation between populations (e.g. Irwin *et al.* 2008; Ballentine *et al.* 2013; Cooney *et al.* 2017).

Gene flow describes the movement of genes between divergent populations and is an important mechanism for divergence and introgression (Morjan and Rieseberg 2004; Slatkin 1977; Slatkin 1987). Speciation is thought to require low gene flow because high gene flow

will generally increase the amount of overlapping genetic variation between populations, leading to introgression. However, gene flow is more frequently being recognised as a process affecting populations that remain divergent (Galligan *et al.* 2012; Peters *et al.* 2017). The geographic landscape may be an important factor that allows populations to diverge in the presence of gene flow under strong natural selection (Butlin *et al.* 2014; Oatley *et al.* 2017; Sulloway and Kleindorfer 2013), or between fragmented habitats (Dudaniec *et al.* 2011; Myers *et al.* 2010; Weir *et al.* 2015). Differences in particular traits known as magic traits may be important in the development of reproductive incompatibility between populations that are not geographically isolated. These traits are simultaneously effected by ecological divergence and assortative mating and allow populations to remain divergent in the absence of a geographic barrier to gene flow (Gavrilets 2004; Servedio *et al.* 2011) with implications for hybrid fitness (Hamilton and Miller 2016; Pfennig *et al.* 2016; Taylor *et al.* 2015). Hybrids that adapt to novel environments could form distinct populations that diverge from their parental lineages (Mallet *et al.* 2007; Seehausen 2004; Taylor *et al.* 2015). When gene flow is extensive between divergent parental lineages, this could lead to species extinction through outbreeding depression or genetic replacement (Frankham 1998). Asymmetric gene flow is a pattern that is often found between divergent populations that interbreed. Some research suggests that patterns of asymmetrical gene flow may also be important in the development of reproductive isolation because it may be linked to reinforcement of reproductive incompatibilities (Yukilevich 2012).

Markers of divergence and introgression

Mapping gene flow between divergent populations is important for elucidating processes related to divergence and introgression, and hence markers of divergence and introgression are useful to examine landscape patterns of variation. There are a range of characteristics

associated with different markers that allow different evolutionary processes to be investigated. For example, markers of divergence that are associated with differences in the environment or sex are likely to be under natural or sexual selection. Nuclear genes could indicate introgression and are useful for detecting individual admixture. Mitochondrial genes are markers of divergence that are useful for assigning individuals to populations and identifying population boundaries. Some populations may be affected by more than one evolutionary process so mapping multiple markers of divergence is often necessary in order to understand interactions between different evolutionary processes.

Novel genetic variants are often associated with unique morphological phenotypes (Abzhanov *et al.* 2006; Hohenlohe *et al.* 2010), and therefore morphological differences may be associated with changes in allele frequencies produced through natural selection or genetic drift (Dowle *et al.* 2015; Johannesson and Butlin 2017; Smith 2011). Within species, morphological divergence is often related to patterns of environmental variation or geographic isolation (Funk *et al.* 2016; Milá *et al.* 2010; Schlotfeldt and Kleindorfer 2006), with greater similarity in morphology when individuals occur in similar environments (Butlin *et al.* 2014). Gene flow between morphologically divergent populations could result in either morphological introgression of one population into another or it could produce novel morphological phenotypes (Keller *et al.* 2013; Leafloor *et al.* 2013). The geographic distribution of different morphotypes could also be affected by the genetic dominance of particular alleles that affect particular morphological traits (Baldassarre *et al.* 2014). Sharp morphological boundaries could reveal potential reproductive barriers between divergent populations or genetic dominance related to a particular morphological trait. Other patterns of morphological divergence may or may not be associated with population divergence and different evolutionary processes (Table 1.1).

Mitochondrial divergence is a good proxy for identifying species and understanding historical processes associated with divergence (Singhal and Moritz 2013). The coalescence times for mitochondrial genes are four times shorter than for nuclear genes (Brown *et al.* 1982). Lineage sorting is more efficient for mitochondrial genes because it is inherited uniparentally (Avise 2009). Nuclear divergence associated with speciation will cause mitochondrial genes to diverge because different species are likely to mate assortatively (Zink and Barrowclough 2008). Geographic isolation will also rapidly cause the divergence of mitochondrial genes even though nuclear genes may remain relatively undiverged (Hickerson *et al.* 2010). As the geographic landscape changes, populations that have diverged in allopatry are able to make secondary contact or alternatively populations may have diverged in the presence of gene flow. Mitochondrial genes are useful for detecting patterns that distinguish between these scenarios (e.g. McLean *et al.* 2017; Sardell and Uy 2016). Differences between the distribution of mitochondrial lineages and patterns of nuclear divergence can lead to inferences about whether contemporary evolutionary processes have been affected by gene flow in the past or present (Endler 1982; Rollins *et al.* 2012; Shipham *et al.* 2015; Toon *et al.* 2010). The distribution of mitochondrial variation therefore can be used to distinguish between 1) low nuclear divergence related to existing high gene flow and 2)

Table 1.1 Sources of variation explained by different mechanisms that may or may not contribute to population divergence.

Sources of variation	Morphological variation	Nuclear genetic variation	Geographical variation	Population divergence	Evolutionary process	Reference
Rare variants	✓	✗	✗	✗	N/A	Donnellan *et al.* (2015); Johannesson and Butlin (2017)
Plasticity/ Epigenetics	✓	✗	✓	✗	N/A	Galligan *et al.* (2012); Gruber *et al.* (2013); Nussey *et al.* (2007)
Convergence/ Trait conservation	✗	✓	✓ or ✗	✓	Natural selection	Butlin *et al.* (2014); Welch *et al.* (2011)
Sympatric divergence/ Discordance	✓	✓	✗	✓	Natural selection, genetic drift, gene flow	Dowle *et al.* (2015); Mebert *et al.* (2015)

low nuclear divergence related to contemporary or weak divergence in previously or presently allopatric populations. Distinguishing between these scenarios is important for understanding factors that affect evolutionary processes.

Identifying patterns of divergence and introgression is important for conservation management as it facilitates the identification of species and areas where individuals are more or less likely to persist (Allendorf *et al.* 2010; Frankham 2010; Steiner *et al.* 2013). The classification of species is dependent on identifying patterns of divergence and reproductive isolation between populations (De Queiroz 2007). Subspecies that are considerably diverged and do not interbreed in the absence of a geographic barrier to gene flow may be taxonomically raised to species. Theoretically, species represent a greater proportion of biodiversity than subspecies because they are more unique (Moritz 1994; Phillimore and Owens 2006; Zink 2004). Therefore, species are more likely to be given a higher conservation status than subspecies. Identifying hybrids is also important for conservation. Hybrid individuals may have a greater chance of persisting because they may have novel adaptations that can cope better in new environments (Hamilton and Miller 2016). Even low introgression can increase the adaptive capacity of a population as they will have greater genetic diversity. Finally, high levels of introgression between divergent lineages can lead to the extinction of a population when that population is genetically swamped by the source population (Price and Muir 2008). Informed conservation management requires studies that identify patterns of divergence and introgression.

*The thick-billed grasswren (*Amytornis modestus*) as a model of divergence and introgression*

The Maluridae are a group of songbirds that are endemic to Australasia and consist of three genera that includes the well-studied and highly diverse fairywrens (*Malurus*). There are eleven species of fairywren that are sexually dimorphic (males are brightly coloured while

females are dull coloured), have complex song and generally occupy mesic habitats, some with overlapping subspecies distributions (Baldassarre *et al.* 2014; McLean *et al.* 2017; Rowley and Russell 1997). Grasswrens (*Amytornis*), another clade of the Maluridae, also contain eleven species but differ noticeably from fairywrens. Male grasswrens are drab and have less colouration than female grasswrens, and they generally occur in small and geographically isolated populations restricted to arid or semi-arid regions (Rowley and Russell 1997). There is a paucity of information on evolutionary processes affecting grasswrens due to the shy nature of grasswrens and their occurrence in areas with low infrastructure. What is currently known about the genera indicates that the drivers of divergence between grasswrens and fairywrens may be different (Buchanan and Cockburn 2013; Christidis *et al.* 2010). For example, fairywrens are socially monogamous but sexually promiscuous (Rowe and Pruett-Jones 2013). Grasswrens are thought to be both socially and sexually monogamous as extra-pair paternity in grasswrens has not been detected (Louter 2016). Investigating the drivers of divergence in grasswrens enables us to understand broader evolutionary patterns associated with this family.

The thick-billed grasswren (*Amytornis modestus*) has a large amount of cladogenesis compared to other grasswren species. There are currently seven recognised subspecies of thick-billed grasswren that are found within the Northern Territory, South Australia and New South Wales (Black 2011; Black 2016). Two of those subspecies are thought to be extinct and four of the remaining extant subspecies hold a conservation status greater than 'of Least Concern' (Black 2016; Garnett *et al.* 2011). All thick-billed grasswren subspecies are known to have allopatric distributions except *A. m. indulkanna* and *A. m. raglessi* are separated by an area that is only 50 km wide. Thick-billed grasswrens are believed to be low dispersing but whether a 50-km gap between the subspecies is enough to prevent gene flow is not currently known. These two thick-billed grasswren subspecies are a good model for understanding

divergence within the Maluridae as their low levels of divergence and near-connectivity allows us to test for mechanisms that influence divergence and introgression.

Thesis aims and objectives

This thesis will assess the processes that affect divergence and introgression between two subspecies of the thick-billed grasswren. Chapter 2 will determine the distribution patterns of the subspecies (*A. m. indulkanna* and *A. m. raglessi*) using morphological and mitochondrial markers. Chapter 3 aims to identify the geographic variation in habitat across the subspecies distributions particularly within the 50-km gap that separates the subspecies. Chapter 4 aims to measure gene flow and admixture in association with landscape features using nuclear genetic markers. Chapter 5 will assess whether differences in territorial behaviour may be affecting introgression and divergence between the subspecies. The main objective of this thesis is to determine what factors are affecting the processes of divergence and introgression in this arid zone songbird species of the Maluridae. This information will broaden our understanding of evolution within the Maluridae and will be used to inform appropriate conservation management strategies.

Organisation of the thesis

This thesis contains a series of manuscripts that are published or in preparation for publication in scientific, peer-reviewed journals. I am the first author for all manuscripts that have been or will be published. Each manuscript forms a separate chapter and is formatted consistently for the purpose of this thesis. Some repetition between data chapters is unavoidable because each data chapter is presented as an individual publication. This thesis contains three published papers (Chapters 2, 3 and 5) and one paper that is in preparation for submission (Chapter 4). I conclude the thesis with a general discussion of the main findings and implications.

Chapter 2 Patterns of morphological and mitochondrial diversity in parapatric subspecies of the thick-billed grasswren (*Amytornis modestus*)

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Abstract

Divergence is the first phase of speciation and is commonly thought to occur more readily in allopatric populations. Subspecies are populations that are divergent but generally retain the capacity to interbreed should they come into contact. Two subspecies of the thick-billed grasswren (*Amytornis modestus*) are divergent by 1.7% at the mitochondrial ND2 gene and were previously considered to be allopatric. In this study, we discovered that the subspecies were parapatric. We use a larger sample size than previous studies to examine variation in morphology and mitochondrial haplotype across the distribution of each subspecies and within the region of parapatry. The subspecies occurring to the west, *Amytornis modestus indulkanna,* had larger body size and longer and narrower bill than the subspecies occurring to the east, *A. m. raglessi*. Within the region of parapatry, females were morphologically similar to *A. m. indulkanna* but had eastern mitochondrial haplotypes while males had intermediate morphology and either eastern or western haplotypes. Additionally, haplotypes from the western mitochondrial clade were found in *A. m. raglessi*. These patterns of morphology and mitochondrial diversity reveal discordance within the region of parapatry and to the east. We suggest that the subspecies have undergone asymmetric expansion from west to east, made secondary contact, and are currently hybridising.

Introduction

The biogeographic landscape frequently drives the evolution of new species and can influence the persistence of biodiversity by affecting population connectivity. Allopatric populations are often associated with divergent evolution (Ford 1987; Grant *et al.* 2000; Norman *et al.* 2007) as they develop different allele frequencies that can rapidly reach fixation in the absence of gene flow (Price 2010; Winger and Bates 2015). Parapatric populations are often established when changing landscapes remove geographic barriers to gene flow and allow allopatric populations to make secondary contact. Parapatric populations with 'porous borders' have an increased probability of hybridising, which may either confer fitness benefits to hybrids by increasing genetic diversity (Rheindt and Edwards 2011; Seehausen 2004) or reduce hybrid fitness when alternative phenotypes are selected against (Alexandrino *et al.* 2005; Dingle *et al.* 2010; Hoskin *et al.* 2005; Laaksonen *et al.* 2015; Schumer *et al.* 2015). Genetically diverse populations are associated with greater persistence because they may have greater adaptive potential (Markert *et al.* 2010; Rheindt and Edwards 2011). Populations with reduced gene flow in the absence of a geographic barrier are associated with speciation according to the Biological Species Concept (Mayr 1942). Divergent populations that are parapatric make good case studies for understanding processes underpinning divergence, as well as for understanding how changes in gene flow can generate diversity and/or cause speciation.

Morphological differentiation is a traditional indicator of population divergence. Natural selection, sexual selection and habitat matching are all considered processes that create morphologically differentiated populations. Adaptive morphological traits under selection generally confer an individual with increased trait utility (e.g. foraging efficiency, mating success) (Ballentine *et al.* 2013; Myers *et al.* 2012; Schlotfeldt and Kleindorfer 2006). Habitats that are highly connected are important for habitat matching as individuals with

particular traits move into habitats where the individual has increased trait utility. Population divergence driven by individuals expressing habitat matching behaviour may be a selective process because phenotypically suited individuals should have a fitness advantage (Webster *et al.* 2012). In the presence of a geographic barrier, morphological divergence is facilitated by genetic drift and impeded gene flow given enough time following vicariance (Gaither *et al.* 2015; Petren *et al.* 2005). When divergent subspecies abut, the morphological pattern across the intervening region is expected to be clinal when there is a low level of gene flow (Baldassarre *et al.* 2014; Sulloway and Kleindorfer 2013). In some cases, morphological differentiation is correlated with an ecological gradient in the presence of high gene flow through phenotypic plasticity (Gruber *et al.* 2013; Kruuk *et al.* 2015). In this case, morphologically differentiated populations will not be genetically differentiated (nuclear markers). An examination of morphotype distribution across the landscape is a good starting point to estimate selective landscapes that could be shaping observed patterns of divergence.

Subspecies are evolutionary lineages that provide insight into the spatio-temporal context of evolutionary outcomes (Byrne 2008; Byrne *et al.* 2008). The thick-billed grasswren (*Amytornis modestus*, TBGW) is a polytypic species that is restricted to the arid zone of inland Australia. The species is considered to be a highly sedentary habitat specialist; these traits make it susceptible to the effects of geographic isolation across heterogeneous landscapes (Rowley and Russell 1997). There are currently seven named subspecies of TBGW that are from geographically isolated regions and/or are assembled into two distinct mitochondrial (mtDNA) clades of the NADH dehydrogenase 2 (ND2) gene (Austin *et al.* 2013; Black 2016). These clades are 1.7% divergent and are thought to have begun divergence during the mid-Pleistocene (~400 kya) (Austin *et al.* 2013). The two subspecies in this study have mtDNA haplotypes either from the eastern or western clade. Morphological analyses of the subspecies to date have been performed using small sample sizes; these

results indicate sexual dimorphism in *A. m. raglessi* (males have longer tails than females) that was not present in *A. m. indulkanna* (Black 2011; Black 2016). The subspecies are considered to be allopatric with respective distributions to the east and west of the South Australian salt lakes, Lake Eyre and Lake Torrens (Black *et al.* 2011). This distribution is associated with divergence across the Eyrean Barrier (Ford 1974; Keast 1961; Serventy 1972), a well-established feature of the biogeographic landscape that has prevented gene flow between isolated populations in the past and prompted the divergence of many avian species (Dolman and Joseph 2015; Joseph and Omland 2009). Today, a dune field that would have been much larger and drier in the past separates the subspecies (Byrne *et al.* 2008). These sand dunes are likely to be associated with the divergence of TBGW subspecies.

This study tests the hypothesis that the geographic distribution of two grasswren subspecies *A. m. indulkanna* and *A. m. raglessi* includes a region of parapatry where birds have not been observed previously (Black *et al.* 2011). Specifically, we will test whether the subspecies (1) differ in morphology and mtDNA haplotype using a larger field-based sample size compared with earlier studies (Austin *et al.* 2013; Black 2011), (2) have a region of morphological and genetic overlap, and (3) interbreed in the region of parapatry. We compare the morphology and mtDNA haplotype of each subspecies per geographical area of its known distribution and then assess the occurrence of morphotypes and haplotypes in the 'intervening zone' – the purported region of parapatry between the documented regions of allopatry known for each subspecies. The location of the intervening zone coincides with Lake Eyre and Lake Torrens, which may have geographically isolated the subspecies in the past. We predict we will find mtDNA haplotypes from both eastern and western mtDNA clades in the region of parapatry, and intermediate morphology.

Methods

Study species

The TBGW is a cursorial songbird that today occurs only in South Australia and New South Wales (Black *et al.* 2011). TBGWs are morphologically divergent from their sister species, the western grasswren (*A. textilis*), as western grasswrens have a longer tail and darker plumage (Black *et al.* 2010). Like most grasswrens, TBGWs are sexually dimorphic in plumage pattern where females have a rufous patch on the flanks and males do not (Rowley and Russell 1997). A consensus regarding the number of subspecies within the TBGW clade has been difficult to obtain because many populations have subtle morphological differences (Black *et al.* 2010; Christidis *et al.* 2010; Christidis *et al.* 2013). While Christidis *et al.* (2013) suggest there are two subspecies of TBGW, we use the taxonomy of Black (2011) and Black (2016) that proposes seven subspecies of TBGW as this has been widely accepted (Gill and Donsker 2017; Skroblin and Murphy 2013). The two subspecies used in this study, *A. m. indulkanna* and *A. m. raglessi,* are of interest because they are geographically proximate (Black *et al.* 2011) and represent reasonably divergent mtDNA clades (Austin *et al.* 2013).

Study sites

This study was conducted in South Australia at 88 study sites (Figure 2.1); the 88 study sites include locations from which DNA samples had previously been collected (Austin *et al.* 2013) as well as new sampling locations. The sampling locations are grouped into three zones defined by the previously known allopatric distribution of the subspecies described in Black *et al.* (2011). Zone AB is located between Lake Eyre and Lake Torrens where grasswrens were detected for the first time and subspecies assignment is unknown, zone A (*A. m. indulkanna*) is to the west of zone AB and zone B (*A. m. raglessi*) is to the east of zone AB.

There is a dune field located between zone A and zone B (Figure 2.1). Study sites were grouped into ~2500 km^2 areas to facilitate the description of the zones. Zone A comprises Andamooka, Coward Springs Railway Siding, William Creek, Peculiar Knob, Coober Pedy, Mount Barry station, Oodnadatta and Oodnadatta East. Zone B comprises Witchelina Nature Reserve, Mount Lyndhurst station and Murnpeowie station. Zone AB comprises Mulgaria station and Stuart Creek station to the east of the Borefield Rd (Figure 2.1).

Samples and sampling method

Our dataset included 154 birds; 2 were missing haplotype sequences and 38 were missing morphometrics. Sample sizes for morphometric data were: zone A, males $n = 23$ and females $n = 13$; zone B, males $n = 40$ and females $n = 30$; zone AB, males $n = 6$ and females $n = 4$. Sample sizes for haplotype data were: zone A, males $n = 30$, females $n = 22$ and one sample with unknown sex; zone B, males $n = 50$ and females $n = 35$; zone AB, males $n = 6$ and females $n = 8$ (see Appendix). We collected all morphometric data as well as 137 blood samples for haplotype data in the field. Additional haplotype data came from one tissue sample from the South Australian Museum, Adelaide and previously published mtDNA haplotype sequences (Austin *et al.* 2013). To find study sites we surveyed areas with previous TBGW sightings as well as other areas with chenopod shrub vegetation cover. In locations where we successfully found grasswrens, we erected between one and four standard mist nets (12 m long and 30 x 30 mm mesh size; Flinders University animal ethics approval #E385). Mist-netting was performed only during daylight hours. Each individual was banded with an Australian Bird and Bat Banding Scheme alloy identification band (licence #2601 and #3108). Blood samples were collected from the brachial vein using a capillary tube or jugular vein using a syringe. Samples were stored in a salt solution preservative (Seutin *et al.* 1991) at 4°C or on specially designed Whatman FTA cards (Sigma-Aldrich Pty Ltd, NSW,

Australia) that allows storage of blood at room temperature while preventing DNA degradation.

Morphometrics

Morphological measurements were recorded from the bill, tarsus, wing and tail. All measurements were taken with dial callipers to the nearest 0.1 mm unless otherwise stated. Bill length was measured from the approximate centre of the right naris to the tip of the bill. Bill depth and bill width were measured at the widest part at the base of the bill. Tarsus (tarsometatarsus) length was measured from the proximal bend of the tibiotarsal articulation to the base of the toes. Wing length was measured from the carpal joint to the tip of the longest primary feather while the wing was flattened and tail length was measured from the base of the tail to the end of the longest central feather. Wing and tail measurements were collected with a ruler to the nearest 1 mm. Mass was measured using a digital scale to the nearest 0.1 g. Inter-observer variation between the two measurers (authors A.L.S. and M.L.) was addressed by assuring high repeatability of measurements until 98% accuracy was achieved during a calibration phase, where each researcher measured the same bird. Where birds were trapped more than once across multiple years, only measurements from the first trapping were analysed. Juveniles that were identified by their yellow gape were excluded from the analysis.

Morphological statistics

Morphometrics from 116 samples were analysed using a principal components analysis (PCA). This is a common method for creating an uncorrelated set of variables for morphological comparisons (Arbeláez-Cortés *et al.* 2014; Sattler and Braun 2000; Smith *et al.* 2011). Factor scores were calculated following promax rotation and Kaiser normalisation with SPSS STATISTICS v22.0 (SPSS Inc., Chicago, USA). Three components were

extracted with a proportion of variance greater than 10% and that cumulatively explained 73.3% of the variance across all samples (Table 2.1). The variables that loaded heavily for component one (PC1, body size, eigenvalue 2.12) were tarsus length, bill length and mass. An increase in PC1 indicates a tendency for body size to be larger and bill length to be longer. Component two (PC2, bill shape, eigenvalue 2.10) loaded heavily for bill width and bill depth. Birds with higher values for component two had wider bills. Component three (PC3, body shape, eigenvalue 0.90) loaded heavily for wing length and tail length. Birds with higher values for component three had longer wings and tail.

Morphological differences between subspecies

Due to a skew in the sample sizes between *A. m. indulkanna* and *A. m. raglessi*, we used a Welch *t*-test for unequal variances (Ruxton 2006) to compare factor scores between zone A and zone B. The subspecies sampled within zone AB was unknown and excluded from this analysis. The factor scores contained no outliers as assessed by inspection of a boxplot for values greater than three box-lengths from the edge of the box and all were normally distributed, as assessed using a Shapiro-Wilk test ($p > 0.05$). We then performed a non-parametric Mann-Whitney *U* test to determine which specific traits were different between *A. m. indulkanna* and *A. m. raglessi*. Distributions of the variables for individuals in zone A and zone B were not similar, as assessed by visual inspection, therefore the Mann-Whitney *U* test was used to test differences in mean ranks rather than medians.

PCR amplification and DNA sequencing

DNA extractions were carried out in a separate pre-PCR laboratory in order to minimise DNA contamination. Different methods of DNA extraction were used for samples stored in different forms. Tissue from museum samples and blood in salt solution were extracted using a DNeasy Blood and Tissue kit (QIAGEN Pty Ltd, VIC, Australia) or a Gentra Puregene

Blood Kit (QIAGEN Pty Ltd, VIC, Australia). Blood stored on FTA card was extracted using the method from Smith and Burgoyne (2004). TBGW-specific primers that target ~800 bp of the mtDNA ND2 gene were designed based on previously published sequences (Austin *et al.* 2013). These primers were: M1684 (5'-ATGGCGAGAATTACAGGGG-3') and M1685 (5'-CCATCACAATTTCAAGCAACC-3'). PCR reactions were performed in 25 µl volumes containing 10-100 ng of extracted DNA, with a final concentration of 1x MRT buffer (1x Immolase buffer, 2 mM $MgCl_2$, 2 mM dATP, 2 mM dCTP, 2 mM dTTP, 2 mM dGTP, 0.5x BSA), 0.2 µM of each primer and 0.5 units Immolase (Bioline (Aust) Pty Ltd, NSW, Australia). PCR cycling conditions were: denaturation at 95°C for 10 mins, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 60 s with a final extension of 72°C for 10 mins. Sanger sequencing was performed after filtering PCR products with a multiscreen PCR plate vacuum cleanup (Millipore Australia Pty Ltd, NSW, Australia).

Sex determination

The sex of an individual was initially determined in the field by the presence or absence of rufous patches on the flanks. We assumed that an individual was female if the rufous patch was present and male if it was absent. Sex was later confirmed using a modified PCR that amplifies a sex-chromosome-related region of the Chromo-Helicase-DNA-binding (CHD) gene (Griffiths *et al.* 1998). The forward primer, P8 (5'-CTCCCAAGGATGAGRAAYTG-3') and reverse primer, P2 (5'-TCTGCATCGCTAAATCCTTT-3') were used in 25 µl reactions containing 10-100 ng of extracted DNA, with a final concentration of 1x MRT buffer, 0.2 µM of each primer and 0.5 units Immolase (Bioline (Aust) Pty Ltd, NSW, Australia). PCR cycling conditions were: denaturation at 94°C for 10 mins, followed by 35 cycles of 94°C for 45 s, 53°C for 30 s and 72°C for 60 s, with a final extension of 72°C for 10 mins. The amplified region on the Z chromosome is smaller than on the W chromosome

and in TBGWs, the homogametic males (ZZ) produced a single fragment of ~360 bp whilst the heterogametic females (ZW) produced two fragments that were ~360 bp and ~400 bp in size. The plumage-based convention of sexing the TBGW was in agreement with genotyping in all cases.

Sexual dimorphism

We tested for sexual dimorphism within zone A (*A. m. indulkanna*) and zone B (*A. m. raglessi*) in order to determine whether the sexes should be analysed separately between zones. The subspecies sampled within zone AB was unknown and excluded from this analysis. We determined whether there was sexual dimorphism by comparing factor scores with an independent *t*-test. The factor scores contained no outliers as assessed by inspection of a boxplot for values greater than three box-lengths from the edge of the box and all factor scores were normally distributed, as assessed using a Shapiro-Wilk test ($p > 0.05$). There was homogeneity of variances for all three PCA factors in zone A and zone B, as assessed by Levene's test for equality of variances ($p > 0.05$). We then used a non-parametric Mann-Whitney *U* test to determine which specific traits were sexually dimorphic. In order to assess differences in medians, variable distributions are required to be similar. This was not the case when comparing zone A and zone B, as assessed by visual inspection, therefore the Mann-Whitney *U* test was used to demonstrate differences in mean ranks rather than medians.

Mitochondrial haplotype statistics

ND2 sequences were aligned, checked and edited using GENEIOUS v8.0.5 (Biomatters, Auckland, NZ). In order to assign mtDNA clade (eastern or western) to samples, sequences were analysed using an unrooted neighbour-joining tree with the Tamura-Nei Model. This tree was then applied as input to generate an unrooted mtDNA haplotype network using HAPLOTYPE VIEWER (Salzburger *et al.* 2011). Four sequence positions with missing data

were excluded from the mtDNA haplotype network. To assess the level of mitochondrial divergence between the mtDNA clades we performed an analysis of molecular variance (AMOVA) with 1000 permutations (Excoffier *et al.* 1992) using the program GENALEX v6.5 (Peakall and Smouse 2012). All consecutive statistical analysis was performed in DNASP v5.10.01 (Librado and Rozas 2009). Genetic variation within the mtDNA clades was assessed using nucleotide diversity (π) and mtDNA clade diversity (Hd). To determine whether selection is affecting divergence between eastern and western mtDNA clades (Pavlova *et al.* 2013), we used a McDonald and Kreitman (MK) test (McDonald and Kreitman 1991). We first tested the complete sequence and then tested sequence positions separated into surface or transmembrane protein domains. This is an effective method for detecting weak selection signals (Zink 2005). To test for population expansion, we performed a Ramos-Onsins and Rozas's R_2 and Fu's F_S test and determined the significance of these values using a coalescent simulation with 5000 replicates (Fu 1997; Ramos-Onsins and Rozas 2002). These tests assume that both subspecies have a large and constant population size and are not under selection and that the genetic sequences are not affected by recombination (Ramos-Onsins and Rozas 2002).

Results

Sexual dimorphism

Sexual dimorphism was present in both zone A and zone B and although patterns of sexual dimorphism were similar between zones for all principal components, sexual dimorphism for individual traits differed between zones (Table 2.2; Table S 2.1). An independent *t*-test showed that all principal components (body size, bill shape and body shape) were sexually dimorphic in both zone A and zone B ($p < 0.05$) (Table 2.2). A Mann-Whitney *U* test showed that within zone B, all individual traits were sexually dimorphic ($p < 0.01$). Within zone A,

bill depth, tarsus length, wing length and tail length were sexually dimorphic ($p < 0.01$) while bill length, bill width and mass were not ($p > 0.05$). Sexually dimorphic traits were larger in males than females in both subspecies (Table S 2.1). Morphological ordination of bill shape (PC2) and body shape (PC3) produced two overlapping clusters associated with males and females within both zones (Figure 2.2).

Morphological differences

Because there were sexually dimorphic traits in both zone A and zone B, males and females were analysed separately when comparing zones. Ordination of body size (PC1) and bill shape (PC2) produced two clusters associated with the geographic areas, zone A and zone B, that were not as closely associated with mtDNA clade (Figure 2.2). The differences between geographic clusters were more distinct for females than males but both sexes followed the same trends. Birds in zone A had a large body and narrow and long bill compared to birds in zone B. Males within zone AB had intermediate morphology with a small body (PC1) and narrow and long bill (PC2) while females within zone AB had morphology typical to females in zone A with a large body and long and narrow bill (Figure 2.2). A Welch *t*-test that compared zone A and zone B showed that all principal components were significantly different in males and females (all $p < 0.05$; Table 2.2). The results were similar when comparing individual traits, which are shown in Table S 2.2. Body size (PC1) was significantly different in males from zone AB compared to zone A ($p < 0.001$) but not zone B ($p = 0.471$) and bill shape (PC2) was significantly different in males from zone AB compared to zone B ($p < 0.001$) but not zone A ($p = 0.748$). Body size (PC1) and bill shape (PC2) were significantly different in females from zone AB compared to zone B (both $p < 0.001$) but neither of these components was significantly different in females from zone AB compared to zone A (PC1: $p = 0.813$, PC2: $p = 0.907$, PC3: $p = 0.158$). Body shape (PC3) was not

significantly different in any comparison between zone A and zone AB or zone B and zone AB.

Genetic differences

We analysed the partial sequence (749 bp) of the mitochondrial ND2 gene from 152 specimens of *A. modestus* including previously published sequences (Austin *et al.* 2013). This sequence started at position 171 and ended at position 920 of the full 1041 bp-long sequence. In total, 41 unique mtDNA haplotypes were detected that were grouped into two mtDNA clades, eastern and western (Figure 2.3). Both mtDNA clades showed high mtDNA haplotype diversity (range 0.85-0.89) and low nucleotide diversity (0.003; Table 2.3). The western mtDNA clade was most prevalent in zone A (100%), but was also found in zone AB (36%) and extended into zone B (11%). The eastern mtDNA clade was found only in zones AB and B (Figure 2.3). Individuals in zone B that had western mtDNA haplotypes were both male and female and these individuals all had morphology typical to zone B individuals (*A. m. raglessi*; Figure 2.2). There were both males and females with eastern mtDNA haplotypes within zone AB; males had an intermediate morphology while females had morphology typical to females in zone A (*A. m. indulkanna*; Figure 2.2). Males in zone AB with a western mtDNA haplotype also had intermediate morphology but morphological measurements from females in zone AB with western mtDNA haplotypes were excluded because the birds were juvenile.

We tested for evidence of selection on the ND2 gene by comparing proportions of fixed and polymorphic substitutions that were synonymous and non-synonymous between the mtDNA clades. There were 2 non-synonymous and 6 synonymous fixed substitutions compared to 8 non-synonymous and 30 synonymous polymorphic substitutions along the 749 bp sequence. Selection on protein coding genes is inferred when there is a difference in the ratio between

fixed substitutions (non-synonymous and synonymous) and polymorphic substitutions (non-synonymous and synonymous) (McDonald and Kreitman 1991). Selection on the ND2 gene was not significant for the 749 bp sequence or for domains associated with transmembrane or surface proteins ($p > 0.05$; Table S 2.3). AMOVA analysis indicated that divergence between eastern and western mtDNA clades accounted for 82% of the total molecular variance (Table 2.4) and these clades had an F_{ST} of 0.823 ($p = 0.001$). Fu's F_S and Ramos-Onsins and Rozas's R_2 were significant for the western mtDNA clade (both $p < 0.008$) but neither test was significant for the eastern mtDNA clade (Table 2.3). This pattern is also reflected in (1) the unimodal mismatch distribution of the western mtDNA clade, which shows a higher frequency of greater pairwise differences than the eastern mtDNA clade, and (2) the shape of the mtDNA haplotype network where there were more low frequency polymorphisms that were adjacent to each other in the western mtDNA clade (Figure 2.3).

Discussion

The results of this study confirm that the mtDNA haplotypes and morphotypes of two TBGW subspecies are divergent (Austin *et al.* 2013; Black 2011), and reveal that the subspecies (1) have a parapatric distribution, and (2) are hybridising within the intervening zone (zone AB). Female grasswrens in zone AB had morphology typical of *A. m. indulkanna* from the west (zone A) but had mtDNA haplotypes from the eastern mtDNA clade. There were two females in zone AB that had mtDNA haplotypes from the western mtDNA clade but the morphological measurements for these individuals were excluded. Males in zone AB had intermediate morphology and mtDNA haplotypes from either eastern or western mtDNA clades. The results also reveal an asymmetric pattern in the distribution of morphotypes and mtDNA haplotypes. Some individuals in the east (*A. m. raglessi*; zone B) had morphology consistent with *A. m. raglessi* but had western mtDNA haplotypes; no individual in the west (*A. m. indulkanna*; zone A) had an eastern (*A. m. raglessi*) morphotype or an eastern mtDNA

haplotype. These patterns suggest that *A. m. indulkanna* underwent population and range expansion across the sand dunes that occur in the intervening area, but there was no range expansion for *A. m. raglessi*. This interpretation is further supported by statistical tests of population expansion on the mtDNA haplotypes. Therefore, we conclude that TBGWs have dispersed from west to east, that the region of parapatry arose from secondary contact within the intervening region, and that the subspecies are interbreeding.

The TBGW subspecies *A. m. indulkanna* and *A. m. raglessi* were likely allopatric in the past but are currently parapatric and without any geographic barrier to gene flow. We suggest this is because the subspecies have made secondary contact in the intervening area. As expected in areas of allopatry with no gene flow, there were clear differences in morphology and mtDNA haplotypes across regions to the east and west of Lake Eyre and Lake Torrens. These two large areas (east and west of the lakes) were adjacent to a dune field associated with the Eyrean Barrier that separated the subspecies in the past (Austin *et al.* 2013). Australia has experienced periods of high aridity during glacial maxima and sand dunes would have been more extensive in the past (Byrne *et al.* 2008). Biogeographic barriers have been used to explain divergence in many other arid-adapted bird taxa (Ford 1974; Keast 1961; Serventy 1972). Within the intervening area between Lake Eyre and Lake Torrens, the pattern was different: no mtDNA clade was dominant and male morphology could not be assigned to either subspecies. We conclude that patterns of divergence between TBGW subspecies are consistent with divergence in allopatry followed by secondary contact.

Some individuals within the intervening area between Lake Eyre and Lake Torrens and to the east of Lake Eyre and Lake Torrens had mtDNA haplotypes that were discordant with morphotype. This indicates that in the absence of a geographic barrier the subspecies do not have a complete reproductive barrier. Joseph and Omland (2009) and McKay and Zink

(2010) suggest a number of processes that cause mitochondrial discordance, such as hybridisation, incorrect taxonomy based on geographic distributions and incomplete lineage sorting (ILS). Assuming that TBGW morphotypes are associated with nuclear genetic divergence, this study removes the possibility of incorrect taxonomy or ILS because (1) all discordant mtDNA haplotypes occurred either within or close to the region of parapatry, (2) there were no unique mtDNA haplotypes in the east that were derived from western mtDNA haplotypes which would be expected in the case of ILS, (3) the discordant mtDNA haplotypes were shared at both internal and external positions on the mtDNA haplotype network rather than only at internal positions such as with ILS, and (4) the inferred mitochondrial divergence time between the mtDNA clades is considerable (~0.36 Mya) (Austin *et al.* 2013). Examples that attribute mtDNA haplotype and morphological discordance to hybridisation between divergent taxa are well documented (Kearns *et al.* 2014; Shipham *et al.* 2015; Weckstein *et al.* 2001). The zone of hybridisation detected in this study appears to be very narrow because the morphological overlap between the east and the west was very small (within zone AB), particularly for females as the morphology of the sampled females was typical of A (*A. m. indulkanna*). Further research that uses a larger sample size within the region of parapatry and tests nuclear genetic divergence is recommended in order to determine the frequency of hybridisation and the extent of introgression (see Chapter 4).

Amytornis modestus indulkanna but not *A. m. raglessi* has experienced a population and range expansion resulting in asymmetric gene flow across the parapatric zone. This is supported by (1) significant R_2 and Fu's F_S values for western mtDNA haplotypes but not eastern mtDNA haplotypes, (2) the unimodal mismatch distribution for western mtDNA haplotypes and a trend for the mismatch distribution of eastern mtDNA haplotypes to be bimodal, (3) the intermediate and western morphology of birds to the east of the suspected

sand dune barrier, and (4) the lack of eastern mtDNA haplotypes to the west of the suspected sand dune barrier. Although the mtDNA haplotype sampling density within zone A (*A. m. indulkanna*) (n = 1-12 per 2500 km^2 area) was much less than for zone B (*A. m. raglessi)* (n = 2-79 per 2500 km^2 area), it is unlikely that this was the reason why eastern mtDNA haplotypes were not detected in the west (zone A). The abundance of western (zone A) mtDNA haplotypes in the east (zone B) was one in eight samples; if we expect to detect eastern (zone B) mtDNA haplotypes in the west (zone A) at a similar sampling density, we should have detected them in at least four of six western locations where the sample size was more than eight. Western mtDNA haplotypes extended further from the western subspecies' range (*A. m. indulkanna*) than did *A. m. indulkanna* morphotypes. Mito-nuclear discordance with deeper mitochondrial introgression is expected in systems where there is selection for particular mito-nuclear combinations or that have female-biased dispersal (Toews and Brelsford 2012). We speculate that the range expansion of *A. m. indulkanna* may be a relatively recent event following considerable divergence of mtDNA haplogroups that may be due to environmental changes that have affected their population numbers or distribution patterns. Further research using nuclear genetic markers is recommended to determine whether female-biased dispersal may be producing asymmetric mtDNA haplotype distributions in TBGW subspecies and whether recent secondary contact between TBGW subspecies may be related to ecological changes in the landscape.

Conclusion

This study examined two TBGW subspecies within their previously known distributions and a region where the subspecies were parapatric and found different patterns of morphology and mtDNA haplotypes. Individuals within the geographical areas associated with the previously known distribution of the two subspecies had different morphology irrespective of

mtDNA haplotype. There was also a high proportion of either the eastern or western mtDNA clade within these geographical areas. Individuals within the intervening parapatric zone were either similar to *A. m. indulkanna* with an eastern mtDNA haplotype or intermediate between *A. m. indulkanna* and *A. m. raglessi* with either eastern or western mtDNA haplotypes. There was an asymmetry in mtDNA haplotype distribution, with greater mitochondrial discordance in *A. m. raglessi* than *A. m. indulkanna*. Combined, these findings enhance our understanding of patterns of subspecies distribution within a region of parapatry.

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Table 2.1 Component coefficients of PCA. Morphological components (PC1: body size, PC2: bill shape, PC3: body shape) were determined using promax rotation and Kaiser normalisation. Variables with the highest factor loadings are shown in bold.

Variable	PC1	PC2	PC3	Communalities
Tarsus	**0.890**	0.162	-0.149	0.729
Mass	**0.803**	0.105	0.051	0.649
Bill length	**0.648**	-0.395	0.131	0.681
Bill width	-0.188	**0.822**	0.012	0.768
Bill depth	0.318	**0.839**	0.058	0.754
Tail length	0.046	-0.101	**0.922**	0.827
Wing length	-0.110	0.256	**0.744**	0.720

Table 2.2 Independent *t*-test statistics comparing PC scores between zones (A, B) and sex (male, female). Comparisons of either sex or zone groups as stated in the first column. Variables where $p < 0.05$ are shown in bold.

Sex or Zone	Group 1	Group 2	Trait	Mean (group 1)	Mean (group 2)	*t*-value	df	*p*-value (2-tailed)
Male	Zone A (*n* = 23)	Zone B (*n* = 40)	PC1: body size	1.014 ± 0.132	0.022 ± 0.126	5.123	61	**0.001**
			PC2: bill shape	-0.743 ± 0.101	0.859 ± 0.107	-9.983	61	**0.001**
			PC3: body shape	0.056 ± 0.149	0.598 ± 0.168	-2.177	61	**0.033**
Female	Zone A (*n* = 13)	Zone B (*n* = 30)	PC1: body size	0.488 ± 0.183	-1.016 ± 0.127	6.622	41	**0.001**
			PC2: bill shape	-1.213 ± 0.136	0.280 ± 0.131	-6.833	41	**0.001**
			PC3: body shape	-1.054 ± 0.142	-0.276 ± 0.137	-3.399	41	**0.002**
Zone A	Male (*n* = 23)	Female (*n* = 13)	PC1: body size	1.014 ± 0.132	0.488 ± 0.183	2.366	34	**0.024**
			PC2: bill shape	-0.743 ± 0.101	-1.213 ± 0.136	2.789	34	**0.009**
			PC3: body shape	0.056 ± 0.149	-1.054 ± 0.142	4.917	34	**0.001**
Zone B	Male (*n* = 40)	Female (*n* = 30)	PC1: body size	0.022 ± 0.126	-1.016 ± 0.127	5.706	68	**0.001**
			PC2: bill shape	0.859 ± 0.107	0.280 ± 0.131	3.460	68	**0.001**
			PC3: body shape	0.598 ± 0.168	-0.276 ± 0.137	3.842	68	**0.001**

Table 2.3 Population genetic statistics for western and eastern mtDNA clades. Abbreviations are: sample size (*N*), number of segregating sites (S), number of mtDNA haplotypes (H), sequence length (L), nucleotide diversity (π), mtDNA haplotype diversity (Hd), mean number of pairwise differences within mtDNA clades (PD), Fu's F_S and Ramos-Onsins and Rozas's R_2 tests followed by their respective *p*-values (in parentheses). Variables where $p < 0.05$ are shown in bold.

Area	*N*	S	H	L (bp)	π[a]	Hd	PD	R_2	Fu's F_S
Western	67	27	26	748	0.003 ± 0.003	0.89 ± 0.03	2.594	**0.0444 (0.022)**	**-18.900 (0.001)**
Eastern	85	16	15	748	0.003 ± 0.002	0.85 ± 0.03	2.322	0.0713 (0.267)	-4.045 (0.068)

[a] With correction (Jukes and Cantor 1969)

Table 2.4 Analysis of molecular variance (AMOVA) between western and eastern mtDNA clades.

Source of variation	df	Sum of squares	Percentage of variation	Variance components
Between clades	1	452.671	82	6.024
Within clades	150	194.112	18	1.294

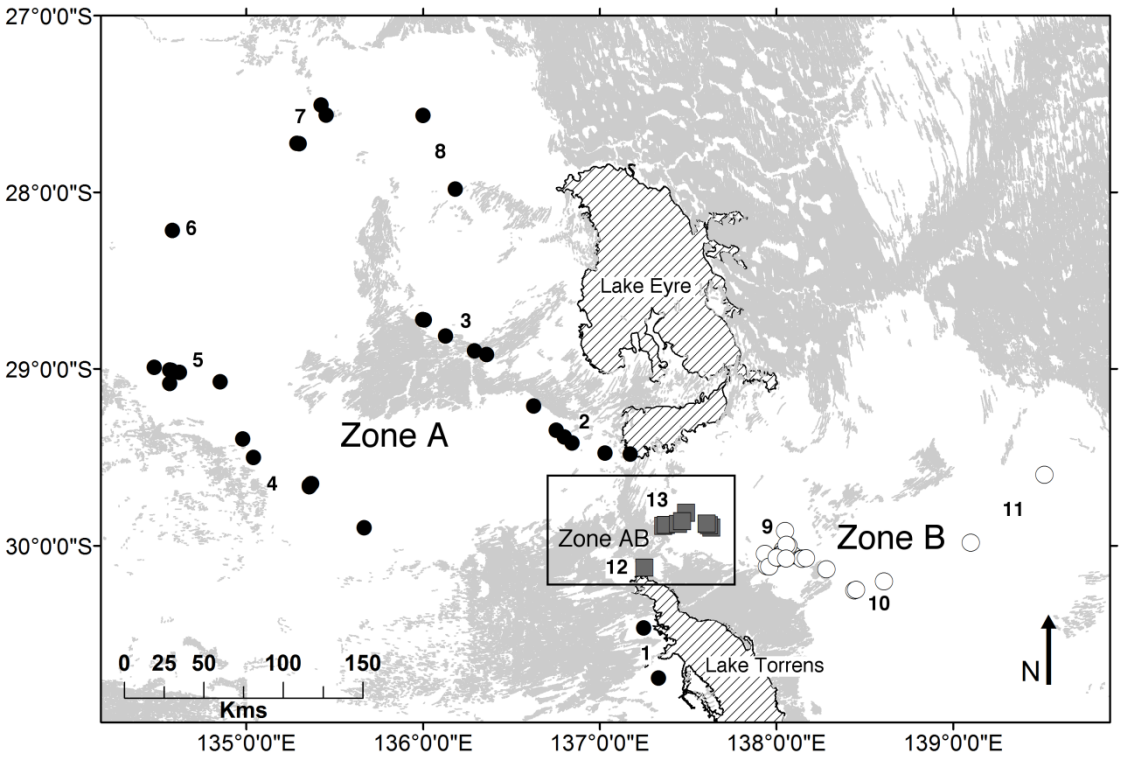

Figure 2.1 A map of the field sites for morphology and haplotype sampling in two thick-billed grasswren (TBGW) subspecies in South Australia. The circles (filled, hollow) indicate sampling locations in the present study in relation to geographical areas of prior known distribution. The squares (grey) indicate sampling locations from an intervening area in which neither subspecies had previously been collected. Study sites occur in zones as follows: zone A (filled circles, *A. m. indulkanna*) includes 1. Andamooka, 2. Coward Springs Railway Siding, 3. William Creek, 4. Peculiar Knob, 5. Coober Pedy, 6. Mount Barry station, 7. Oodnadatta, and 8. Oodnadatta East; zone B (open circles, *A. m. raglessi*) includes 9. Witchelina Nature Reserve, 10. Mount Lyndhurst station, and 11. Murnpeowie station; zone AB (shaded squares, unknown subspecies), includes 12. Mulgaria station, and 13. Stuart Creek station to the east of the Borefield Rd. The area covered by sand dunes (shaded in grey) separates the sample sites in zone AB from the sample sites in zone A. The presence of TBGWs in Andamooka (zone A) was known prior to this study hence they have been placed in zone A. The morphological measurements of these individuals were not included in this

study and are therefore not likely to influence the level of morphological divergence between the zones. The samples contain western mtDNA haplotypes but subspecies designation of these samples will depend on further genetic analysis. We hypothesise these samples will contain a nucelar genome that resembles the eastern subspecies (*A. m. raglessi*) concordant with allopatric divergence across the predicted sand dune barrier.

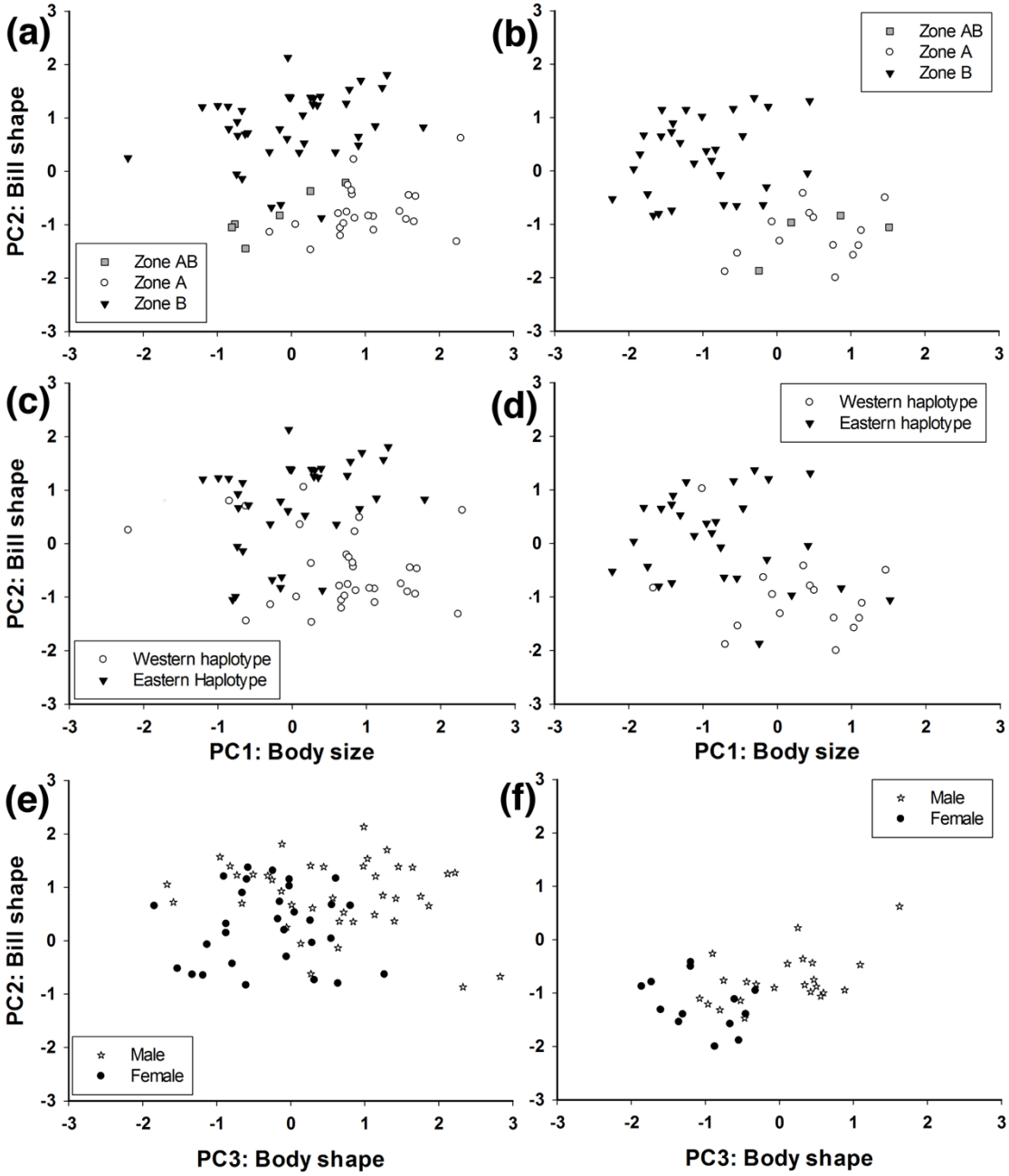

Figure 2.2 Top and middle graphs show ordination of PC1: body size and PC2: bill shape into geographic clusters for (a) males and (b) females and mtDNA haplotype clusters for (c) males and (d) females. Open circles: zone A or *A. m. indulkanna*, black triangles: zone B or *A. m. raglessi*, and grey squares: zone AB. Bottom graphs show ordination of PC2: bill shape and PC3: body shape by sex for (e) zone B (*A. m. raglessi)* and (f) zone A (*A. m. indulkanna)*. Stars: male, and filled circles: female.

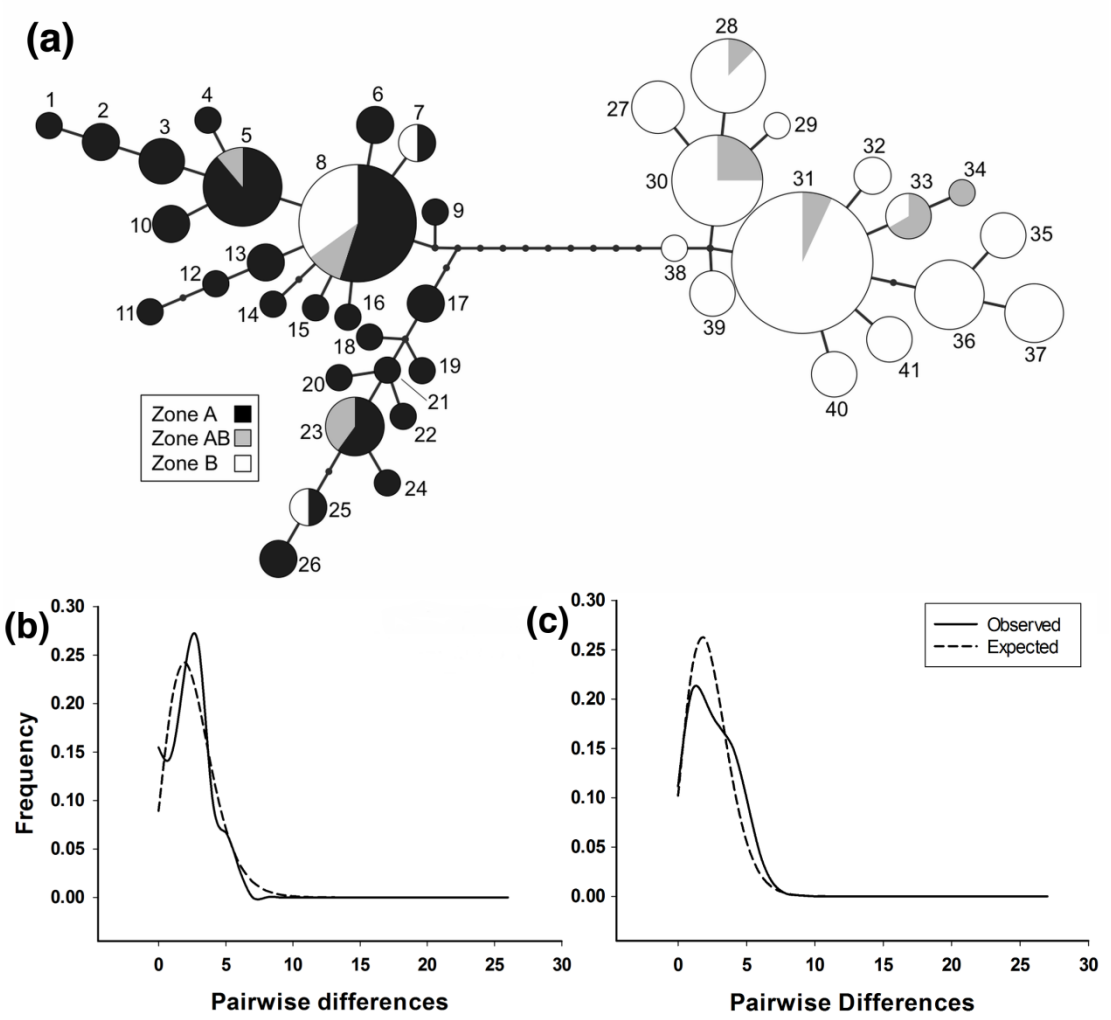

Figure 2.3 (a) An unrooted mtDNA haplotype network for the ND2 gene observed in two thick-billed grasswren subspecies showing the western (left) and eastern (right) clades. Circle diameter reflects mtDNA haplotype frequency. Numbers relate to mtDNA haplotype in Appendix. The shading is the relative frequency of each mtDNA haplotype in zone A (black), zone AB (grey) and zone B (white). Mismatch distributions for the (b) western mtDNA clade and (c) eastern mtDNA clade. The x-axis is the number of pairwise differences between haplotypes; the y-axis is the frequency. The unbroken line indicates the observed frequency and the dashed line indicates the expected frequency calculated by DNASP (Librado and Rozas 2009).

Table S 2.1 Descriptive statistics comparing morphological traits for differences between zones within sex groups. Mann-Whitney *U* test statistics comparing traits (*n*, mean ± s.e.) between zone A and zone B and the proportion of difference (D) between zone A and zone B. Traits where $p < 0.05$ are shown in bold.

Sex	Character	Zone A (*A. m. indulkanna*)		Zone AB (Parapatry)		Zone B (*A. m. raglessi*)		D (%)	Mean Rank (Zone A)	Mean Rank (Zone B)	Mann-Whitney *U* score	*z*-score	*p*-value
Male	Bill length (mm)	24	8.2±0.1	6	7.7±0.1	41	7.5±0.1	**8.5**	48.56	23.89	118.5	-5.090	**0.001**
	Bill width (mm)	24	5.3±0.0	6	5.4±0.1	42	6.2±0.1	**10**	14.65	44.27	956.5	6.046	**0.001**
	Bill depth (mm)	24	5.9±0.1	6	5.7±0.1	42	6.2±0.0	**4.8**	21.25	40.56	800.5	3.978	**0.001**
	Tarsus length (mm)	24	25.2±0.1	6	23.7±0.3	42	24.3±0.1	**3.8**	45.33	26.74	220	-3.788	**0.001**
	Wing length (mm)	24	59.0±0.3	6	58.2±0.5	42	60.5±0.3	**2.5**	24.31	38.75	724.5	2.981	**0.003**
	Tail length (mm)	23	70.0±0.5	6	70.5±1.8	41	71.6±0.6	2.2	26.52	35.85	609	1.932	0.053
	Mass (g)	24	20.0±0.3	6	19.2±0.4	42	19.6±0.2	2.0	36.27	31.92	437.5	-0.887	0.375

Female	Bill length (mm)	15	7.9±0.1	4	7.9±0.1	31	7.1±0.1	**10.1**	36.57	17.18	36.5	-4.604	**0.001**
	Bill width (mm)	15	5.3±0.1	4	5.3±0.1	31	6.0±0.1	**11.7**	11.23	29.44	416.5	4.321	**0.001**
	Bill depth (mm)	14	5.5±0.1	4	5.5±0.0	31	5.9±0.1	**6.8**	13.82	27.15	345.5	3.165	**0.002**
	Tarsus length (mm)	15	24.4±0.2	4	24.4±0.4	31	23.6±0.1	**3.3**	33.37	18.73	84.5	-3.471	**0.001**
	Wing length (mm)	14	56.4±0.3	4	57.0±1.2	31	59.3±0.4	**4.9**	10	28.87	399	4.505	**0.001**
	Tail length (mm)	14	67.1±0.6	4	69.1±1.2	31	68.2±0.6	1.6	19.64	24.52	264	1.160	0.246
	Mass (g)	15	19.8±0.5	4	20.9±0.9	30	18.0±0.2	**9.1**	31.37	18.82	99.5	-3.024	**0.002**

Table S 2.2 Descriptive statistics comparing morphological traits for sexual dimorphism within subspecies. Mann-Whitney U test statistics comparing traits (n, Mean ± S.E.) and the proportion of difference (D) between groups. Traits where $p < 0.05$ are shown in bold.

Subspecies	Character	Male		Female		D (%)	Mean Rank (Male)	Mean Rank (Female)	Mann-Whitney *U* score	*z*-score	*p*-value
A. m. indulkanna (Zone A)	Bill length (mm)	24	8.2±0.1	15	7.9±0.1	3.7	22.58	15.87	118	-1.798	0.072
	Bill width (mm)	24	5.3±0.0	15	5.3±0.1	0	19.12	21.40	201	0.612	0.541
	Bill depth (mm)	24	5.9±0.1	14	5.5±0.1	**6.8**	23.1	13.32	81.5	-2.632	**0.008**
	Tarsus length (mm)	24	25.2±0.1	15	24.4±0.2	**3.2**	24.21	13.27	79	-2.918	**0.004**
	Wing length (mm)	24	59.0±0.3	14	56.4±0.3	**4.4**	25.5	9.21	24	-4.421	**0.001**
	Tail length (mm)	23	70.0±0.5	14	67.1±0.6	**4.1**	23.39	11.79	60	-3.190	**0.001**
	Mass (g)	24	20.0±0.3	15	19.8±0.5	1.0	20.46	19.27	39	0.751	0.751

A. m. raglessi (Zone B)	Bill length (mm)	41	7.5±0.1	31	7.1±0.1	**5.3**	42.71	28.29	381	-2.902	**0.004**
	Bill width (mm)	42	6.2±0.1	31	6.0±0.1	**3.2**	42.63	29.37	414.5	-2.649	**0.008**
	Bill depth (mm)	42	6.2±0.0	31	5.9±0.1	**4.8**	44.95	26.23	317	-3.751	**0.001**
	Tarsus length (mm)	42	24.3±0.1	31	23.6±0.1	**2.9**	45.06	26.08	312.5	-3.781	**0.001**
	Wing length (mm)	42	60.5±0.3	31	59.3±0.4	**2.0**	42.68	29.31	412.5	-2.689	**0.007**
	Tail length (mm)	41	71.6±0.6	31	68.2±0.6	**4.7**	44.2	26.32	320	-3.599	**0.001**
	Mass (g)	42	19.6±0.2	30	18.0±0.2	**8.2**	45.85	23.42	237.5	-4.486	**0.001**

Table S 2.3 Comparison of nucleotide differences between western and eastern mtDNA clades. Comparisons for the complete sequence region (749 bp) as well as regions related to transmembrane and surface proteins. McDonald and Kreitman (MK) test demonstrates evidence for selection. NS = not significant.

Sequence region	Number of codons	Fixed synonymous	Fixed non-synonymous	Polymorphic synonymous	Polymorphic non-synonymous	MK *p*-value
Complete	245	6	2	30	8	NS
Transmembrane	158	4	1	19	6	NS
Surface	87	2	1	11	2	NS

Chapter 3 Plant community predicts the distribution and occurrence of thick-billed grasswren subspecies (*Amytornis modestus*) in a region of parapatry

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Abstract

Habitat heterogeneity can have considerable effects on gene flow and migration across a region of parapatry. Describing habitat across a region of parapatry is important for the development of eco-evolutionary theory. Two subspecies of thick-billed grasswren (*Amytornis modestus indulkanna and A. m. raglessi*) share a region of parapatry between the South Australian salt lakes, Lake Eyre and Lake Torrens. While the two subspecies remain morphologically distinct outside the region of parapatry, it is not known what factors within the region of parapatry may affect migration and gene flow. In this study, we test associations between habitat differences, connectivity and population divergence to determine whether ecological barriers could play a role in mitigating gene flow between the subspecies. We compare dominant plant species (1) between the ranges of the subspecies and within their region of parapatry, and (2) in relation to presence or absence of grasswrens within their region of parapatry. We found that the dominant plant communities differed between grasswren subspecies and in the region of parapatry. Within the region of parapatry, the dominant plant communities differed at sites with or without grasswrens. Specifically, grasswren absence was associated with the plant communities found on sand dunes. These

findings are discussed in light of evidence for secondary contact and hybridisation between *A. m. indulkanna* and *A. m. raglessi*, and susceptibility to introgression.

Introduction

Divergent evolution in ecologically distinct environments is an important component of speciation (Schluter 2001; Schluter and Grant 1984b). Ecologically heterogeneous landscapes are often linked to population divergence because they provide a mechanism for inhibiting gene flow between populations. For example, fragmented habitats can act as barriers to gene flow by preventing dispersal between populations (Amos *et al.* 2012; Dudaniec *et al.* 2011), and ecologically distinct habitats may promote selection for different traits that lower the probability of interbreeding (Ravinet *et al.* 2016). When diverged populations come into contact, gene flow may be enhanced through hybridisation, or reduced when reproductive barriers are reinforced. In either case, parapatric populations may develop novel evolutionary trajectories given (1) the formation of novel phenotypes through hybridisation (Kleindorfer *et al.* 2014b), (2) genetic introgression via hybridisation (Bierne *et al.* 2013; Borge *et al.* 2005), (3) divergence as a result of selection (Chapman *et al.* 2016) and/or (4) the expression of reproductive barriers that finalise the speciation process (Beysard and Heckel 2014). Despite widespread evidence for the idea that different ecological environments select for different phenotypes, it is not well understood how ecological factors contribute to the occurrence of diverged populations within a region of parapatry. Contemporary ecological landscapes could hinder or favor particular patterns of gene flow affecting processes that lead to speciation.

Plant species with heterogeneous distributions across the landscape can reduce population connectivity among specialist organisms. There is growing evidence that fragmented habitats can disrupt fine-scale population processes such as mate choice or create isolated populations with limited gene flow at larger spatial scales (Athrey *et al.* 2012; Harrisson *et al.* 2013).

Gene flow among populations that are specialists in different habitats may be reduced even when there are no other barriers to gene flow between the ecosystems (Cicero 2004). Some ecosystems are particularly prone to develop heterogeneous ecological landscapes. For example, the Australian arid zone has irregular soil types and limited and sporadic rainfall that creates patchy distributions of different plant communities (Tongway and Ludwig 1990). Animals that live in the arid zone may therefore be affected by both reduced dispersal due to fragmented habitat or specialisation. This will have serious implications for conservation if the distribution of different plant communities is affected by changing climate (Martin 2006). Patchy plant distributions across the Australian arid zone are likely to affect gene flow between populations, even when the populations are in contact. Understanding how habitat changes across the landscape and whether regions of parapatry are associated with distinct habitat types clarifies how the ecological landscape can affect gene flow.

The thick-billed grasswren (TBGW), *Amytornis modestus* is a cursorial songbird that today occurs in the arid zone of South Australia and New South Wales (Black *et al.* 2011). In a previous study, we found that two subspecies of the TBGW (*A. m. indulkanna* and *A. m. raglessi*) are currently parapatric between two South Australian salt lakes; Lake Eyre and Lake Torrens (Slender *et al.* 2017; Chapter 2). These subspecies are morphologically distinct to the east (*A. m. raglessi*) and west (*A. m. indulkanna*) of the salt lakes and are 1.7% genetically diverged at the mitochondrial (mtDNA) ND2 gene (Austin *et al.* 2013; Black *et al.* 2011). *Amytornis modestus raglessi* is currently listed as vulnerable to extinction due to habitat loss and fragmentation (Garnett *et al.* 2011)..Within the predicted region of parapatry, males had intermediate morphology and females had discordant morphotype and mtDNA haplotype indicating that the subspecies may be interbreeding. Most grasswren species (*Amytornis*) are habitat specialists, whereby different grasswrens occur in particular plant communities with a specific vegetation structure (Rowley and Russell 1997). TBGWs are

mostly found in low shrublands containing chenopod shrubs such as *Atriplex nummularia omissa* and *Maireana aphylla* (*A. m. indulkanna*), and *M. pyramidata* and *Rhagodia spinescens* (*A. m. raglessi*) (Black *et al.* 2011). Within the region of parapatry, there is a dune field that stretches between Lake Eyre and Lake Torrens. Apart from this, it is not known what other habitat types occur within the region of parapatry. Describing the ecological landscape within the region of parapatry is important for understanding how gene flow may be affected by eco-evolutionary processes.

This study aims to describe the habitat within the predicted region of parapatry between *A. m. indulkanna* and *A. m. raglessi*. We hypothesise that there is an association between habitat type and grasswren subspecies in the region of parapatry. We make a priori predictions about the location of the region of parapatry based on previous descriptions of TBGW morphology and mtDNA haplotype (Slender *et al.* 2017; Chapter 2) and the core areas of the subspecies based on the known subspecies distributions (Black *et al.* 2011). We then compare (1) plant communities between the subspecies outside the region of parapatry, (2) plant communities within and outside the region of parapatry, and (3) plant communities in the region of parapatry at sites with and without grasswrens.

Methods

Study species and sites

The TBGW is an arid-zone songbird of the Maluridae family (Rowley and Russell 1997). They are one of eleven species of grasswren (reviewed in Skroblin and Murphy 2013), which are endemic to Australia and are well known for their restricted geographic distributions (short range endemism) typical of poorly dispersing organisms (Rowley and Russell 1997). There are seven subspecies of TBGW that are morphologically different (Black 2011; Black

2016). Although not visually distinguishable in the field, *A. m. indulkanna* have a larger body and longer and narrower bill compared to *A. m. raglessi.* Males that occur within the region of parapatry have intermediate morphology, whereas females have morphology similar to *A. m. indulkanna* (Slender *et al.* 2017; Chapter 2). There are two mitochondrial clades (eastern and western) of the NADH dehydrogenase 2 (ND2) gene (Austin *et al.* 2013). There is discordance between mitochondrial clade and morphology within the region of parapatry and within the core area for *A. m. raglessi* (Slender *et al.* 2017; Chapter 2).

The two TBGW subspecies occur only in South Australia; *A. m. indulkanna* is found to the west of Lake Eyre and Lake Torrens and *A. m. raglessi* is found to the east. Because the distribution of each subspecies is so large, data collection was focused within nine ~2500 km^2 areas that cover the subspecies range. Data was collected at a total of 104 sites across these areas (Table 3.1). These areas are used to delimit three zones (Figure 3.1) and to determine the dominant plant species within each zone (described below). Zones represent the previously known distribution of the subspecies as described by Black *et al.* (2011) (zone A and B) and the recently discovered region of parapatry as described by Slender *et al.* (2017; Chapter 2) (zone AB). Zone A includes five areas associated with *A. m. indulkanna* (n = 32 sites), zone B includes two areas associated with *A. m. raglessi* (n = 43 sites), and zone AB includes two areas in the region of parapatry (n = 29 sites) (Figure 3.1). In zone AB, data were collected from sites where TBGWs were present (n = 14) or absent (n = 15).

Thick-billed grasswren occurrence

Collecting data associated with TBGWs is particularly demanding because the TBGW occurs in remote areas with limited infrastructure, and grasswrens are shy and difficult to observe (Black *et al.* 2010; Rowley and Russell 1997). Surveys to record TBGW occurrence were performed from 2012 to 2014, within the months from July to October. A peak in TBGW

breeding activity has been recorded during these months in previous years (Black *et al.* 2011). Observations of TBGW absence were only recorded in zone AB because we were interested in potential habitat fragmentation only within the region of parapatry. Our survey effort was targeted at sites where there was (1) a previous sighting (zone A and zone B), (2) suitable habitat (zone A, zone B and zone AB), or (3) at regular distance intervals (zone AB). Sites with suitable habitat contained > 10 plants within one hectare that were a chenopod shrub species and that were larger than 0.5 m in either height or diameter. This description is based on habitat features previously described for TBGWs (Black *et al.* 2011). In large areas of suitable habitat and in zone AB, sites were sampled by stopping roughly every 10 km along preselected roads or opportunistically if grasswrens were observed from the road.

We surveyed each site by visually scanning the ground and shrubs that were up to 200 m away with binoculars and recording whether grasswrens were seen or heard. We did this while systematically approaching large shrubs in order to flush concealed TBGWs. The survey was focused within one hectare and lasted 60 min. Surveys were conducted on wind-still days (< 15 knots) between 6.30 am (sunrise) and 6:00 pm (sunset), excluding the hotter hours of the middle of the day. Following TBGW surveys, we additionally used playback of TBGW song recordings at sites where grasswrens were not observed. Using a subset of data that were collected in 2014 and that excluded opportunistic sightings, grasswren presence was confirmed by visual or aural detection on average (± SD) 27 ± 24 mins (n = 33) from commencement of the survey. At sites where TBGWs were present, we defined the core territory as the location where (1) there was a nest; (2) there was more than one independent grasswren sighting or (3) a grasswren was first seen. Because most sites were visited multiple times and at least one visit was performed just after sunrise, this proved to be a good estimate of core territory as the same core territories were identified during a radio telemetric study of TBGW home range at Witchelina Nature Reserve (Louter 2016).

Vegetation sampling

We measured vegetation parameters from 2013 to 2015. Vegetation parameters were not likely to change significantly over this time period, as the target plant species (long-lived perennial chenopod shrubs) are slow growing, even in the absence of grazing (Crisp 1978; Crisp and Lange 1976; Osborne *et al.* 1935). Plants were measured across a 400 m^2 area to account for the spatial heterogeneity of arid-zone vegetation that occur across patchy soil types and under conditions of variable rainfall (Morton *et al.* 2011; Williams 1982). We used Jessup transect methodology along a 4 x 100 m transect line that covered a total area of 400 m^2 (Jessup 1951). For ease of measurement, the transect line was segmented into forty 5 x 2 m sample quadrants. We targeted the core of a TBGW territory, which is estimated to be 2 ha (Louter 2016), by placing the centre of the transect within the territory core, as defined above. Two transect arms proceeded 50 m north and 50 m south from the territory center. The cover of all plants (not defined by species) as well as the cover of adult long-lived perennial shrubs (defined by species) was recorded. Adult chenopod shrubs were regarded as plants taller or wider than 10 cm. The variables recorded were plant species (for long-lived perennial shrub species), percent cover of each target plant species and percent cover of all vegetation.

Statistical comparison of dominant plant species

The dominant plant species were identified separately for each independent variable (zone or TBGW presence). Dominant plant species within the presence variable (present/absent) were only analysed within zone AB and dominant plant species within the zone variable (zone A, zone B and zone AB) were only analysed across transects where TBGWs were present. Within each site (400 m^2), the percent cover of a plant species was calculated as a proportion of the total plant cover. The average percent cover of each plant species within an area (2500

km^2) was then calculated. Only plant species with an average cover greater than 1% within an area grouped by the independent variable (zone or presence) were retained for further analysis. Dominant plant species were defined as plant species that were present in more than half the areas within an independent variable group (zone or presence).

To compare the dominant plant species between (1) zones and (2) presence/absence categories, we performed a one-way MANOVA with a post-hoc Tukey test and calculated 95% confidence intervals with 1000 bootstraps. Many samples had zero percent cover of a particular plant species, so the data were inversely transformed before the analysis. The transformed variables still contained some statistical outliers, so we also performed a non-parametric Mann Whitney *U* test to determine the sensitivity of the results. The MANOVA (and confirmatory Mann Whitney *U* tests) were used to compare the percent cover of dominant plant species between (1) zone A and zone B (presence only), (2) zone A and zone AB (presence only), (3) zone B and zone AB (presence only) and (4) presence versus absence (zone AB only). To reduce over-parameterisation of the data, we also calculated derived factor scores with a principle component analysis (PCA) using the percent cover of the dominant plant species with varimax rotation. We performed an independent *t*-test to compare factor scores across the same four tests as above. All statistics were performed using SPSS Statistics v22.0 (SPSS Inc., Chicago, USA).

Results

Variable reduction

Three components were extracted from a PCA of eight plant species that were identified as dominant in any of the independent variables (Table 3.2). These components all had an eigenvalue greater than one that cumulatively explained 63.7% of the variation within the

dataset. The plant species that loaded heavily for component one (PC1, eigenvalue 2.27) were *Atriplex vesicaria* and *Zygochloa paradoxa*. Lower PC1 scores indicated more percent cover from *A. vesicaria* and less percent cover from *Z. paradoxa*. The plant species that loaded heavily for component two (PC2, eigenvalue 1.54) were *Maireana aphylla*, *M. astrotricha* and *M. pyramidata*. Lower PC2 scores indicated more percent cover from *M. astrotricha* and *M. pyramidata* and less percent cover from *M. aphylla*. The plant species that loaded heavily for component three (PC3, eigenvalue 1.28) were *A. nummularia* ssp. *omissa*, *Acacia* spp. and *Rhagodia spinescens*. A lower PC3 score indicated more percent cover from *Acacia* spp. and *R. spinescens* and less percent cover from *A. n. omissa*.

Dominant plant species associated with subspecies

In zone A and zone B (TBGWs present), there were five dominant plant species. The plant species *A. vesicaria*, *M. aphylla*, *M. astrotricha* and *R. spinescens* were dominant in both zone A and zone B, while *M. pyramidata* was only dominant in zone B. Post-hoc comparisons of the MANOVA showed that percent cover in three of five dominant plant species was significantly different between zone A and zone B (Table 3.3). Zone A had more cover from *M. aphylla,* but less cover from *M. astrotricha* and *M. pyramidata* compared with zone B. The plant species *A. vesicaria* and *R. spinescens* had comparable cover in zones A and B. We found similar statistical outcomes using the non-parametric Mann Whitney *U* test, but with an additional significant difference showing more cover from *R. spinescens* in zone A than zone B (three of five comparisons $p < 0.005$, *R. spinescens* $p = 0.017$ and *A. vesicaria*, $p = 0.100$). To control for the effects of possible co-varying plant species, we explored associations between zone and plant species using derived factor scores. We found the same pattern whereby the cover of dominant plant species in zone A and zone B was different. All of the three derived factor scores for cover of dominant plant species were

statistically different between zone A and zone B, PC1 (t (73) = 2.168, p = 0.033), PC2 (t (73) = 5.207, $p < 0.001$) and PC3 (t (73) = 4.929, $p < 0.001$), (Figure 3.2).

Dominant plant species comparison between subspecies and the region of parapatry

In zone AB (TBGWs present), there were four dominant plant species. These included *A. vesicaria*, *M. astrotricha* and *M. pyramidata* (also dominant in zone A and/or zone B) and *A. n. omissa,* which was only dominant within the region of parapatry (zone AB). Two plant species that were only dominant in zone A and zone B, *M. aphylla* and *R. spinescens,* were also included in the analysis. The MANOVA test showed there was variation in cover between five dominant plant species but not for *A. vesicaria* (Table 3.3). Post-hoc comparisons showed there was more *A. n. omissa* in zone AB than either zone A or zone B. Zone A had more cover from *M. aphylla* than zone AB while zone B had more cover from *M. pyramidata*. Of the dominant species, 4/6 (67%) had similar cover between zone A and zone AB, and 4/6 (67%) had similar cover in zone B and zone AB. Apart from one plant species, the non-parametric tests showed a similar result to the MANOVA for comparisons between zone A and zone AB. There was less cover from *M. astrotricha* in zone A than zone AB (all $p > 0.10$ except *A. n. omissa, M. aphylla and M. astrotricha* $p < 0.05$). Apart from one plant species, the non-parametric test also showed similar results to the MANOVA for comparisons between zone B and zone AB. There was more cover from *R. spinescens* in zone B than zone AB (all $p > 0.10$ except *A. n. omissa, R. spinescens* and *M. pyramidata*, $p < 0.01$). The derived factor scores were significantly different between zone A and zone AB for PC2 (t (44) = 3.458, p = 0.001), and between zone B and AB for PC1 (t (55) = -3.031, p = 0.004) and PC3 (t (55) = -4.966, $p < 0.001$) (Figure 3.2).

Dominant plant species associated with occurrence in the region of parapatry

There were six dominant plant species in zone AB where TBGWs were either present or absent. The plant species *M. astrotricha* was dominant in both present and absent groups. The plant species *Acacia* spp. and *Z. paradoxa* were only dominant in the absent group. The plant species *A. n. omissa, M. pyramidata* and *A. vesicaria* were only dominant in the present group. The MANOVA test showed there was more cover from *Z. paradoxa* and less cover from *A. vesicaria, M. pyramidata, M. astrotricha* and *A. n. omissa* where TBGWs were absent compared to where they were present (Table 3.4). The non-parametric test showed similar results except there was no difference in cover of *M. astrotricha* and *A. n. omissa* between presence and absence (three of six comparisons $p > 0.05$ and *A. vesicaria, M. pyramidata* and *Z. paradoxa* $p < 0.05$). Only the derived factor score, PC1, was significantly different between presence and absence (t (23.860) = -3.886, $p < 0.001$) (Figure 3.3).

Discussion

The habitats occupied by each grasswren subspecies (*A. m. indulkanna* and *A. m. raglessi*) and birds in the region of parapatry differed with respect to dominant plant species. Each subspecies core area had different dominant plant species and the region of parapatry contained a mixture of dominant plant species that were also present in either core area. These findings suggest that both *A. m. indulkanna* and *A. m. raglessi* could occur within the region of parapatry, which remains to be tested, because habitat within this region is suitable for both subspecies. The distribution of TBGWs in the region of parapatry was patchy, and we did not detect grasswrens at 52% of surveyed sites. In the region of parapatry, sites without grasswrens had more cover from *Z. paradoxa*, a plant species that occurs mostly along sand dunes. Therefore, sand dunes and their associated vegetation may be a barrier to gene flow between the two TBGW subspecies.

The TBGW subspecies *A. m. indulkanna* and *A. m. raglessi* were found in habitat with different dominant plant species – a finding that mirrors a previous study (Black *et al.* (2011). Black *et al.* (2011) used logistic regression to predict the presence/absence of grasswren subspecies in relation to plant community characteristics. Similar to Black *et al.* (2011), we found that sites with *A. m. raglessi* had greater cover from *M. pyramidata* and *M. astrotricha* whereas sites with *A. m. indulkanna* had greater cover from *M. aphylla* . Subspecies associations with different plant species may be due to heterogeneous plant distributions. Vegetation surveys show that *M. aphylla* and *M. pyramidata* occur in different regions of the Stony Deserts as they belong to different floristic groups (1998). Plant species distributions may limit grasswren species geographical range. For example, the geographic range of the carpentarian grasswren (*Amytornis dorotheae*), which is highly associated with the occurrence of *Triodia* spp (Perry *et al.* 2011), may be limited by the distribution of this plant species. Shifting habitat preference for different plant species has been previously implicated in the divergence of grasswren species. There is a phylogenetic component to the occurrence of different grasswren species in habitat with and without *Triodia* (Christidis *et al.* 2010). The divergence of chenopod shrub species is thought to have provided a new ecological opportunity for TBGWs when they diverged from their sister species, the western grasswren (*Amytornis textilis*), which retains the ancestral habitat type of *Acacia* and *Eucalyptus* (Norman and Christidis 2016). Estimates place the time to most recent common ancestor between the eastern and western TBGW mitochondrial ND2 clades in the Pleistocene (Austin *et al.* 2013; Norman and Christidis 2016). It is possible that contemporary divergence of TBGW subspecies, as evidenced by different morphology (Slender *et al.* 2017; Chapter 2), may be affected by the availability of different chenopod shrubs throughout the range of different grasswren subspecies through a process involving ecological opportunity (e. g. Wellborn and Langerhans 2015).

Habitat clines create opportunity for hybridisation between parapatric populations. In this study, there were different dominant plant species associated with the region of parapatry (zone AB) that were found in the range of *A. m. indulkanna* (*A. m. omissa*) or *A. m. raglessi* (*M. astrotrica*). We previously suggested that the two TBGW subspecies have made secondary contact and are potentially interbreeding in the region of parapatry (Slender *et al.* 2017; Chapter 2). The correlation between the location of the region of parapatry and the presence the vegetation types associated with both subspecies suggest that ecological selection linked with particular plant species could have played a role in maintaining the hybrid zone between the two subspecies (e. g. Endler 1977). Correlations between environmental clines and the presence of hybrid zones are common in other parapatric species and are generally facilitated by low dispersal (Hollander *et al.* 2015; Patel *et al.* 2015) and/or low hybrid fitness (Alexandrino *et al.* 2005; Beysard and Heckel 2014). The distribution and availability of particular plant species may be an important factor mediating TBGW subspecies distribution and gene flow across the region of parapatry.

The fragmentation of particular plant communities may restrict dispersal of TBGW subspecies. In this study, the plant community in the region of parapatry at sites without grasswrens had significantly more *Z. paradoxa* than sites with grasswrens. The arid-adapted *Z. paradoxa* is a specialised plant that is adapted to resource-poor and highly variable (stressful) environments such as sand dunes (Moseby *et al.* 1999; Roda *et al.* 2013). We previously showed that grasswrens with *A. m. indulkanna* morphology and western mitochondrial haplotypes were found east of the dune fields near the region of parapatry, but *A. m. raglessi* morphotypes and eastern mitochondrial haplotypes were missing on the western side of the dune fields (Slender *et al.* 2017; Chapter 2). The findings presented here suggest that *Z. paradoxa* may prevent dispersal of *A. m. raglessi* to the west but not *A. m. indulkanna* to the east. This could lead to greater introgression into *A. m. raglessi.* We

suggest that *A. m. indulkanna* is more tolerant of habitat with *Z. paradoxa*. The extinct sister lineage to *A. m. indulkanna*, *A. m. modestus,* was known to occur in habitat with *Z. paradoxa* (Black 2016; Black 2012) and there have been opportunistic observations of *A. m. indulkanna* in *Z. paradoxa* near the southern shoreline of Lake Eyre South (A. Black, personal communication, 2016). We suggest that *A. m. indulkanna* rarely occupies areas dominated by *Z. paradoxa* but *Z. paradoxa* may allow dispersal of *A. m. indulkanna* through this habitat type. It is possible that we have underestimated the occurrence of TBGWs due to low detectability. However, we suggest that grasswrens that do occur in *Z. paradoxa* are more likely to be *A. m. indulkanna* because they are adjacent and occur to the east and west of the sand dunes. Asymmetric contact zones are common across avian populations with secondary contact (Beysard *et al.* 2015; Dingle *et al.* 2010; Greig and Webster 2013) and may be influenced by variable habitat distribution (Miller *et al.* 2014; Saura *et al.* 2014). Further studies that measure gene flow between the subspecies will reveal the extent of introgression between TBGW subspecies (see Chapter 4).

A number of grasswren species contain populations that are in decline. *Amytornis* contains the highest proportion of threatened species and extinct subspecies within the Maluridae family (reviewed in Skroblin and Murphy 2013). Habitat loss and fragmentation caused by overgrazing from livestock and feral herbivores are likely to be contributing factors (Garnett *et al.* 2011; Reid and Fleming 1992; Schodde 1982). Anthropogenic changes to the landscape may be restricting the geographic range of TBGW subspecies. Plant species respond differently to grazing, which can result in some plant species being more abundant and others less abundant (Fuflendorf *et al.* 2001; Navarro *et al.* 2006). In this study, the region of parapatry and the core area for *A. m. raglessi* were associated with plant species (*M. pyramidata*) that prefer soil disturbed by grazers (Facelli and Springbett 2009). These 'increaser' plant species may have become more abundant since livestock grazing was

introduced to the Stony Deserts over 100 years ago. The plant species *A. n. omissa*, which was found in the core area of *A. m. indulkanna* and in the region of parapatry, has low palatability to livestock and is likely to have become a dominant species after the introduction of feral grazers (Jessop 1995). Other plant species associated with grasswren habitat such as *A. vesicaria* (found in the core areas of both *A. m. raglessi* and *A. m. indulkanna*) and *M. astrotricha* (core area of *A. m. raglessi*) are likely to decrease in the presence of grazing because they are highly palatable or sensitive to grazing (Jessop 1995). Grasswrens occur in habitat that has not been heavily grazed (Louter 2016) and *A. m. raglessi* is currently listed as vulnerable to extinction due to a reduction in available habitat (Garnett *et al.* 2011). Most of the arid rangelands of South Australia are used for pastoralism and are currently grazed extensively by domestic livestock. Conservation management for *A. m. raglessi* should focus on land-management practices and reducing the negative impacts of livestock grazing on long-lived slow growing chenopod shrublands, which are critical for TBGW habitat.

Understanding associations between habitat parameters and gene flow across the landscape is important to conserve populations and interpret evolutionary patterns (James *et al.* 1995). The TBGW is an important model for (1) planning conservation strategies because there are populations that are vulnerable to extinction and (2) understanding factors that affect hybridisation because there are subspecies that are parapatric. This study showed that gene flow across a region of parapatry may be affected by the distribution of different dominant plant species. The absence of a habitat barrier preventing dispersal between subspecies may increase the chance of introgression in the vulnerable TBGW subspecies, *A. m. raglessi*. Gene flow between subspecies that make secondary contact may depend on a pattern of ecological stepping-stones across fragmented habitat and ecological clines within a region of parapatry.

Acknowledgements

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Table 3.1 Coordinates and number of vegetation transects done within each area in zone A, zone B and zone AB, where thick-billed grasswrens were either present or absent (ND = not done).

Site	Latitude	Longitude	Zone	Present (*n*)	Absent (*n*)
Coward Springs Railway Siding	29°24'S	136°49'E	A	7	ND
William Creek	28°54'S	136°20'E	A	6	ND
Peculiar Knob	29°39'S	135°22'E	A	7	ND
Coober Pedy	29°01'S	134°45'E	A	7	ND
Oodnadatta	27°34'S	135°27'E	A	5	ND
Stuart Creek Station	29°43'S	137°04'E	AB	10	11
Mulgaria Station	30°05'S	137°34'E	AB	4	4
Witchelina Nature Reserve	30°01'S	138°03'E	B	36	ND
Mount Lyndhurst Station	30°11'S	138°43'E	B	7	ND

Table 3.2 Variable reduction for dominant plant species. The loading and model contribution of the percentage plant cover of eight dominant shrub species across three zones where thick-billed grasswrens were present or absent. Factor scores were calculated using inverse transformed variables with varimax rotation. Shrub species with the highest factor loadings are shown in bold.

Shrub species	PC1	PC2	PC3	Communalities
Zygochloa paradoxa	**-0.850**	0.059	0.131	0.744
Atriplex vesicaria	**0.677**	0.141	0.084	0.486
Maireana aphylla	0.233	**-0.799**	0.136	0.711
M. astrotricha	0.434	**0.786**	0.066	0.810
M. pyramidata	0.458	**0.597**	0.401	0.727
Rhagodia spinescens	0.120	-0.236	**0.731**	0.604
Acacia spp	-0.295	0.014	**0.726**	0.614
Atriplex nummularia omissa	-0.105	-0.225	**-0.581**	0.399

Table 3.3 Vegetation cover (mean ± s.e. [95% confidence interval]) for six dominant shrub species in Zone: A (n = 32), zone AB (n = 14) and zone B (n = 43), where thick-billed grassrens were present in South Australia. Vegetation cover was calculated using survey transects. The data are shown as raw variables with statistical analysis of the transformed variables using MANOVA. Significant p-values (< 0.05) are shown in bold.

Shrub species	Zone A (%)	Zone AB (%)	Zone B (%)	F	p-value	Partial η^2
Atriplex nummularia omissa[2, 3]	5.44 ± 2.31	6.71 ± 2.37	0.00 ± 0.00	14.1	**0.001**	0.247
	[1.58, 10.82]	[2.51,11.43]	[0.00, 0.00]			
Atriplex vesicaria	5.39 ± 1.35	7.66 ± 2.90	6.84 ± 1.09	1.918	0.153	0.043
	[2.89, 7.93]	[2.33, 13.55]	[4.70, 9.21]			
Maireana aphylla[1, 2]	10.46 ± 2.32	0.21 ± 0.21	2.58 ± 0.85	8.194	**0.001**	0.160
	[6.19, 15.33]	[0.00, 0.72]	[1.11, 4.33]			
M. astrotricha[1]	3.33 ± 1.31	13.90 ± 5.17	8.32 ± 1.61	10.722	**0.001**	0.200
	[1.13, 6.06]	[4.52, 23.94]	[5.51, 12.02]			
M. pyramidata[1, 3]	2.48 ± 1.27	3.29 ± 1.76	12.40 ± 1.77	34.280	**0.001**	0.444
	[0.42, 5.34]	[0.69, 7.69]	[8.85, 15.91]			
Rhagodia spinescens	3.04 ± 1.42	0.54 ± 0.38	2.17 ± 0.49	3.598	**0.032**	0.077
	[0.75, 5.86]	[0.03, 1.39]	[1.28, 3.15]			

1 = zone A is statistically different from zone B (p < 0.05) (Tukey's post-hoc test)
2 = zone A is statistically different from zone AB (p < 0.05) (Tukey's post-hoc test)
3 = zone B is statistically different from zone AB (p < 0.05) (Tukey's post-hoc test)

Table 3.4 Vegetation cover (mean ± s.e. [95% confidence interval]) for six dominant shrub species in zone AB (intervening zone) where thick-billed grasswrens were present (n = 14) or absent (n = 15). Vegetation cover was calculated using survey transects. The data are shown as raw variables with statistical analysis of the transformed variables using MANOVA. Significant p-values (< 0.05) are shown in bold.

Shrub species	Presence (%)	Absence (%)	F	p-value	Partial η^2
Atriplex nummularia omissa	6.71 ± 2.37 [2.25, 11.77]	1.80 ± 1.36 [0.51, 4.91]	5.318	**0.029**	0.165
Atriplex vesicaria	7.66 ± 2.90 [2.33, 14.25]	3.48 ± 2.71 [0.21, 9.46]	7.604	**0.010**	0.220
Maireana astrotricha	13.90 ± 5.17 [4.71, 24.76]	5.57 ± 4.04 [1.85, 14.87]	4.349	**0.047**	0.139
Acacia spp	0.75 ± 0.62 [0.00, 2.09]	3.37 ± 1.89 [0.75, 7.29]	2.651	0.115	0.089
M. pyramidata	3.29 ± 1.76 [0.68, 7.03]	0.00 ± 0.00 [0.00, 0.00]	10.769	**0.003**	0.285
Zygochloa paradoxa	2.15 ± 2.15 [0.00, 7.51]	20.36 ± 6.09 [9.01, 32.68]	8.874	**0.006**	0.247

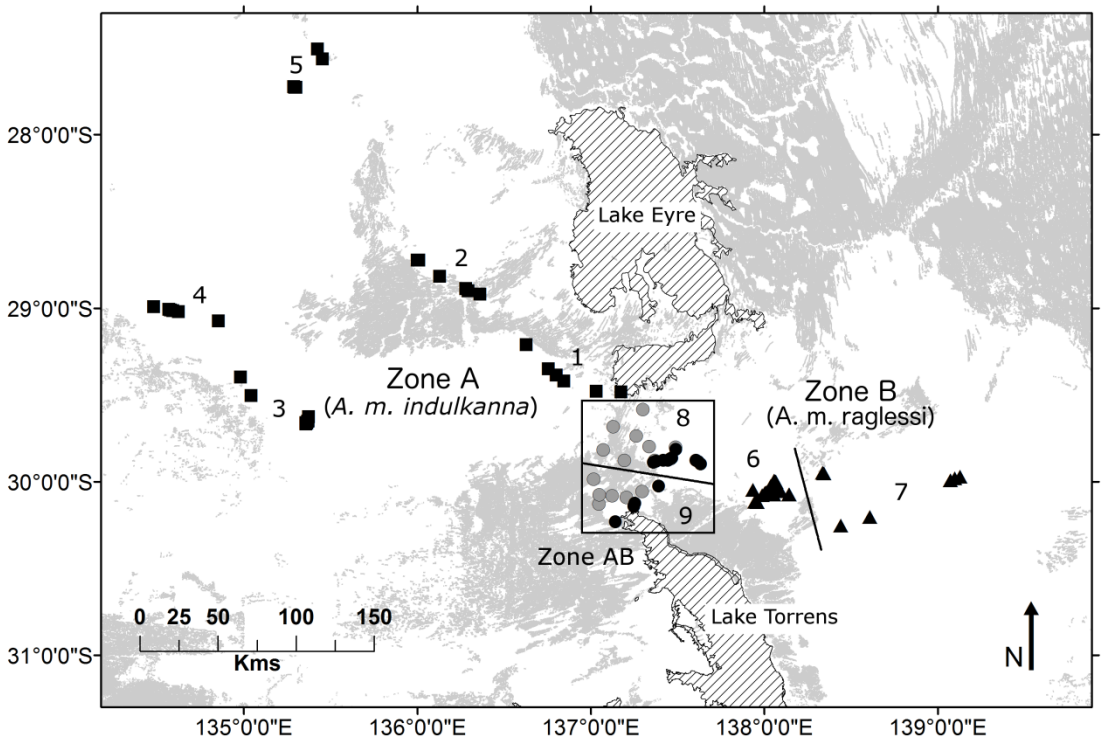

Figure 3.1 Map showing the location of sites where vegetation was sampled for *A. m. indulkanna* and *A. m. raglessi* and their region of parapatry in South Australia. Other thick-billed grasswren subspecies are excluded. Symbols indicate sites where thick-billed grasswrens were present (black) or absent (grey). Sample sites where grasswrens were absent were only measured in zone AB. Sand dunes have been surveyed in locations indicated by light grey shading. Zones (different symbols) are described as 2500 km^2 areas that comprise of multiple sampling sites. Square: Zone A (1: Coward Springs Railway Siding, 2: William Creek, 3: Peculiar Knob, 4: Coober Pedy and 5: Oodnadatta), Circle (black or grey): Zone AB (8: Stuart Creek Station and 9: Mulgaria Station separated by line) and Triangle: Zone B (6: Witchelina Nature Reserve and 7: Mount Lyndhurst Station separated by line).

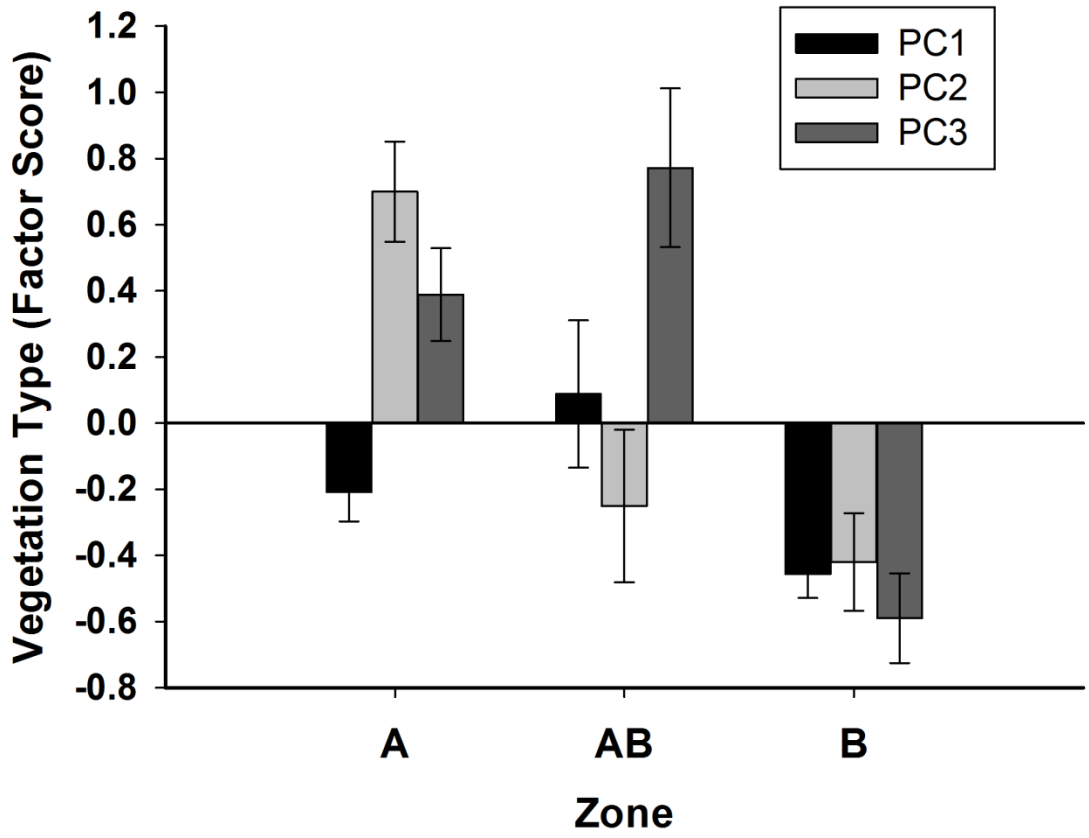

Figure 3.2 Factor scores (mean ± s.e.) representing different plant communities across zones. The derived factor scores (see Table 3.2) are for *Atriplex vesicaria* and *Zygochloa paradoxa* (PC1), *Maireana* spp (PC2) and *Atriplex nummularia omissa*, *Rhagodia spinescens* and *Acacia* spp (PC3). All plant communities (PC1, PC2, PC3) differed significantly between zone A and zone B. Two plant communities (PC1, PC3) differed between zone B and AB, while zone A only differed from zone AB in one plant community (PC2).

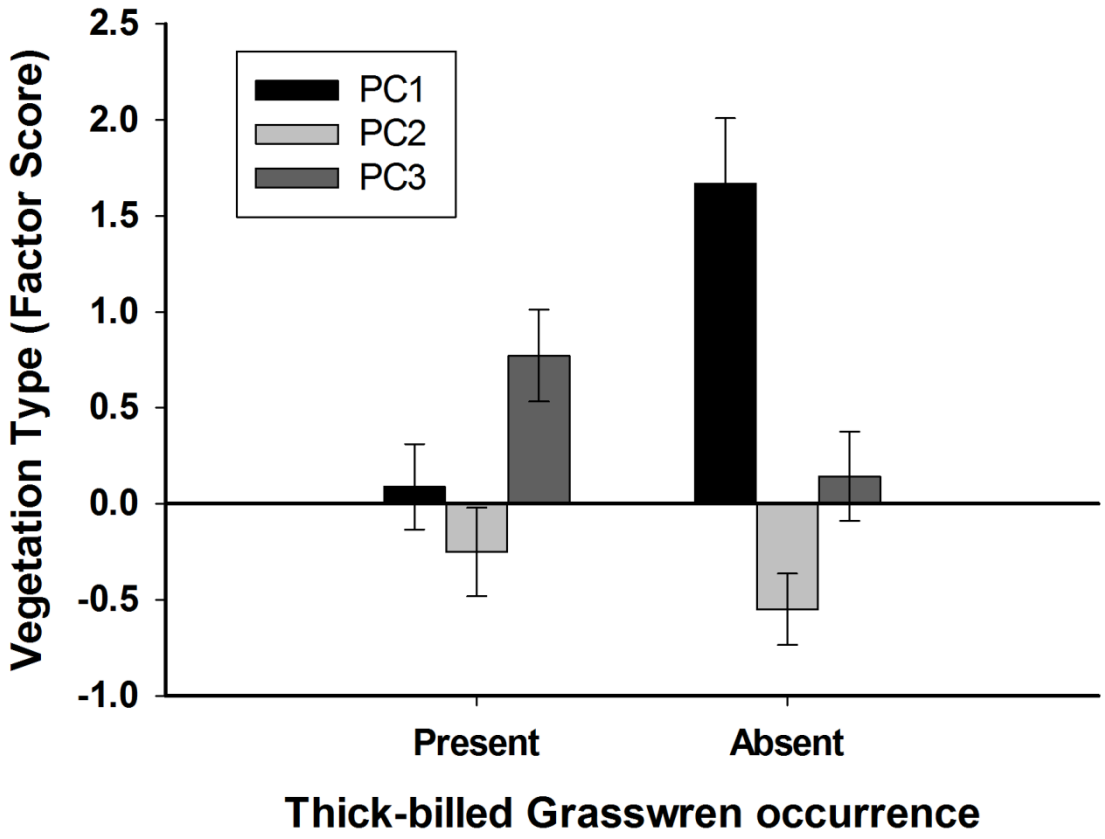

Figure 3.3 Factor scores (mean ± s.e.) representing different plant communities where thick-billed grasswrens are present or absent in zone AB. The derived factor scores for the plant communities with *Atriplex vesicaria* and *Zygochloa paradoxa* (PC1) differed significantly in zone AB where thick-billed grasswrens were present or absent while the derived factor scores for the plant communities with *Maireana* spp (PC2) and *Atriplex nummularia omissa*, *Rhagodia spinescens* and *Acacia* spp (PC3) were similar.

Chapter 4 A heterogeneous landscape produces variable patterns of inter-subspecific gene flow in the thick-billed grasswren (*Amytornis modestus*)

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Abstract

Populations that live in habitat with high spatial heterogeneity are expected to have more gene flow and reduced local adaptation. This study asks whether habitat heterogeneity across a region of parapatry is likely to counteract divergence between two subspecies of thick-billed grasswren (*Amytornis modestus*). These subspecies have two divergent mitochondrial lineages, are morphologically distinct and their ecotypes are different in the area of their core distribution. The subspecies core distributions are connected by a region of parapatry that contain mixed ecotypes where the diverged mitochondrial haplotype of the west extends into the east suggesting landscape heterogeneity may facilitate inter-subspecific gene flow. Using 7583 SNPs, we identified two genetic clusters separated by the area of mixed ecotypes. Limited gene flow was detected in either subspecies core distribution, where the ecotype was distinct. High admixture was present within the area of mixed ecotypes. There were 39 outlier loci associated with the subspecies core distributions, 16 of which were correlated to the abundance of particular plant communities. We detected asymmetric gene flow from west to east and an association between gene flow strength and ecotype distinctiveness. This suggests patterns of divergence between thick-billed grasswren subspecies may be shaped partially by increased gene flow across a heterogeneous landscape and diversifying selection

in different ecotypes. A restricted region of ecological heterogeneity may be important for divergence with gene flow between thick-billed grasswren subspecies.

Introduction

According to the Biological Species Concept, speciation involves the development of reproductive isolation between two related lineages (Mayr 1942). Therefore, identifying species boundaries is dependent on whether gene flow is detected between divergent populations. Different subspecies that have high levels of gene flow across a large geographic area have very ambiguous boundaries and should not be classified as different subspecies (Phillimore and Owens 2006; Zink 2004). Landscape features, such as topography or changes in habitat, may reduce gene flow by preventing dispersal, influencing population demography, or selecting for individuals with particular phenotypes (Beheregaray *et al.* 2015; Goldberg and Lande 2007). Habitat configuration is also important for predicting gene flow (Forester *et al.* 2016). Populations that occur across a habitat gradient will have reduced gene flow because individuals are more likely to disperse within similar habiat and genes associated with local adaptation have low fitness in alternate habitat types (Cicero 2004; Fedorka *et al.* 2012). Populations that occur in more complex habitat configurations, where habitat distributions are heterogeneous, are more likely to have high gene flow (Forester *et al.* 2016). Hybridisation can also facilitate dispersal by creating unique trait combinations that have high fitness outside the habitat type where the populations originated (Pfennig *et al.* 2016). Identifying gene flow and mapping the strength of gene flow across the landscape is useful for determining population boundaries.

Divergence with gene flow is increasingly being recognised as a mechanism for the evolution of species (Gagnaire *et al.* 2015; Nosil 2008; Oswald *et al.* 2017). It can occur between populations that have diverged without any history of allopatry, that are allopatric but have

small amounts of migration, or that have been allopatric in the past but have made secondary contact. Populations with different demographic histories may have different indicators of divergence with gene flow (Cruickshank and Hahn 2014). Populations that are divergent with gene flow but have a history of allopatry or reduced migration may be more likely to be divergent at a limited number of loci or multiple linked loci (Cooney *et al.* 2017; Feder *et al.* 2012; Servedio 2009; Smadja and Butlin 2011). Populations that have strong ecological interactions may be more likely to diverge at multiple unlinked loci (Arnegard *et al.* 2014; Kulmuni and Westram 2017). High migration rates will affect divergence with gene flow as it is predicted to increase the time it takes to accumulate enough genetic incompatibilities that lead to speciation (Yamaguchi and Iwasa 2017). Gene flow may have different effects on populations with different demographic histories and is likely to be an important evolutionary process.

Parapatric populations are good models for studying divergence with gene flow because they are usually examples of early divergence that can be used to test how different factors affect hybridisation (Guiller *et al.* 2017; Niemiller *et al.* 2008; Rabone *et al.* 2015). Parapatric populations will vary in the amount they are differentiated, the amount of diversity they contain and have different interactions with their environment (Barton 2001). These features are likely to determine whether gene flow will cause populations to introgress, remain distinct for long periods of time, or potentially speciate. Parapatric populations that are substantially diverged or occur across very different habitats are likely to form narrow hybrid zones and quickly develop reproductive isolation (Beysard and Heckel 2014; Singhal and Moritz 2013). Gene flow is more likely to be extensive between populations that are less diverged or that occur in similar environments (Harrison and Larson 2016; Rabone *et al.* 2015). Mapping patterns of gene flow between parapatric populations is useful for understanding processes that facilitate divergence with gene flow.

The thick-billed grasswren (*Amytornis modestus ,* TBGW) is an arid-zone species of the Maluridae family that include two subspecies that share a regon of parapatry (*A. m. indulkanna* and *A. m. raglessi*). These subspecies have different morphology and ecotype in the core areas of their distribution (Slender *et al.* 2017; Chapter 2; Chapter 3). The subspecies core areas are located on either side of the salt lakes, Lake Eyre and Lake Torrens; the core area for *A. m. indulkanna* occurs to the west and the core area for *A. m. raglessi* occurs to the east (Black *et al.* 2011; Slender *et al.* 2017; Chapter 2), These subspecies have two divergent mitochondrial (mtDNA) ND2 lineages with a net nucleotide divergence of 1.7% (Austin *et al.* 2013). A number of characteristics related to the distribution of these lineages suggest the subspecies were allopatric in the past (Slender *et al.* 2017; Chapter 2; Chapter 3). We previously showed that these subspecies are currently parapatric and likely to be hybridizing as there was discordance between morphology and haplotype, and the morphology of male birds found between the subspecies core areas was intermediate (Slender *et al.* 2017; Chapter 2). TBGW's were likely to be absent from a dune field that stretches across the region of parapatry (Chapter 3), which may have acted as a barrier to gene flow between the subspecies in the past.

The two TBGW subspecies of this study are a model for testing evolutionary patterns because we can look for associations between gene flow and landscape heterogeneity across a region of parapatry. The ecotype and morphotype of the two TBGW subspecies were distinct in their core distributions, while the region of parapatry contained a complex ecotype with mixture of dominant plant communities (Chapter 3). This study tests the hypothesis that gene flow between the two TBGW subspecies, *A. m. indulkanna* and *A. m. raglessi* will increase across the region of parapatry because the landscape is more complex. We will estimate the extent of gene flow between these subspecies and the distribution of admixed individuals to determine whether patterns of gene flow across the region of parapatry are related to

landscape heterogeneity. We predict (1) population structure will be associated with areas where there is a single dominant ecotype and (2) that high gene flow will occur in areas where the ecotype is heterogenous.

Materials and Methods

Samples

We used a combination of 104 contemporary samples and 14 museum samples collected in South Australia from the TBGW (see Appendix). Contemporary samples were collected in the field by mist-netting birds during the breeding seasons from 2012 to 2015. For further details on the study species and contemporary sample collection methods see Slender *et al.* (2017; Chapter 2). Museum samples were collected from two time periods; four museum samples were from 1985 (*A. m. raglessi*) and the remainder were from 2007 to 2009 (*A. m. raglessi* [$n = 2$] and *A. m. indulkanna* [$n = 8$]). Samples were organized into three geographically associated zones described in Slender *et al.* (2017; Chapter 2) (Figure 4.1). Zone AB includes a region of parapatry that occurs immediately east of a dune field that stretches between the salt lakes, Lake Eyre and Lake Torrens; zone B occurs to the east of zone AB; zone A occurs to the west of zone AB and the dune field. Zone A birds have morphology typical of the subspecies *A. m. indulkanna*, while zone B birds have morphology typical of the subspecies *A. m. raglessi*. Birds in zone AB had either morphology similar to *A. m. indulkanna* (females) or morphology that was intermediate between the two subspecies (males). Two museum samples (SAMA B55668 and SAMA B55667) were formerly included in zone A (Slender *et al.* 2017; Chapter 2) as they were previously thought to be from *A. m. indulkanna* (Black *et al.* 2011); for this study we have extended the boundary between zone A and zone AB so that zone AB includes all samples to the east of the sand dunes. As a result these two museum samples now fall within zone AB.

DNA extraction

Genomic DNA was extracted from tissue and salt solution samples using a DNeasy Blood and Tissue kit (QIAGEN Pty Ltd, VIC, Australia) or a Gentra Puregene Blood Kit (QIAGEN Pty Ltd, VIC, Australia). Genomic DNA was extracted from FTA samples following Smith and Burgoyne (2004). DNA extractions were carried out in a separate PCR free laboratory in order to minimise DNA contamination. DNA quantity was measured using the Qubit fluorometer (ThermoFisher Scientific Australia Pty Ltd, VIC, Australia). DNA extractions were quality tested using UV-spectrophotometry and agarose gel electrophoresis. Samples were assessed as good quality when they showed 1) a large un-degraded band on an agarose gel and 2) a 260/280 ratio between 1.8 and 2.0 indicating minimal protein and chemical contamination.

Library construction and sequencing

A genotyping-by-sequencing library was generated following the protocol in Poland *et al.* (2012). DNA samples (200 ng) were digested with 8 U of PstI and MspI at 37°C for 2 hrs. Each sample was prepared for multiplexing by ligating a pair of adapters containing a unique barcode to the DNA fragments. We used a library of 96 unique barcodes where the barcodes ranged from 4 to 9 bp (Elshire *et al.* 2011). One barcode in each library was assigned as a negative control and seven barcodes in each library were used to duplicate samples within (6 samples) and across (1 sample) libraries. Barcodes were randomly allocated to samples from different geographic locations so that we could detect errors caused by mismatched barcodes that can be made during library preparation or subsequent demultiplexing. We used an adapter mix to DNA ratio of 1:50 ng as this concentration produced libraries with reduced adapter dimer (Elshire et al. 2011). Libraries were then amplified using PCR with the following standard Illumina primers: P5 (5'-

AATGATACGGCGACCACCGAGATCTACAC-3') and P7 (5'-CAAGCAGAAGACGGCATACGAGAT-3'). Sequencing was performed on an Illumina next-seq sequencer that produced single end-reads of 62 bp after adapter trimming. Sequencing data was quality checked using FastQC v10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). For details on methods of haplotype sequencing for the mtDNA ND2 gene see Slender *et al.* (2017; Chapter 2).

SNP calling and filtering

Initially, read filtering and SNP calling was performed using STACKS v1.44 (Catchen *et al.* 2013). Samples were demulitplexed using the *process_radtags* program and reads from sample replicates were merged into one sample (after preliminary SNP calling with separated duplicates to determine error rates). Reads were identified if the adapter barcode (with a maximum of 2 mismatches) and the unique barcode (with a maximum of 1 mismatch) were present. Putative alleles were identified from a stack assembly created with the *ustacks* program that was instructed to include loci with a minimum depth of coverage of 5 reads, maximum distance of 2 nucleotides, and maximum number of 50 stacks per locus. The *cstacks* program was used to create a catalog for identifying loci with a maximum of 2 mismatches between putative alleles. SNPs were determined by comparing the output of *ustacks* with the output of *cstacks* using the *sstacks* program. Relaxing the error tolerance rate improves the likelihood of detecting heterozygous calls (Hohenlohe *et al.* 2010; Lu *et al.* 2013). We used a bounded model for detecting SNPs with the lower error limit of 0.0001 and an upper error limit of 0.05. Minor alleles with low frequency cause problems in population genetics because they can represent sequencing error and they are not informative population markers (Gonçalves da Silva *et al.* 2015). We removed loci if (1) they were present in no more than 20% of individuals, or (2) the minor allele frequency was < 0.05. An individual

was considered heterozygous at a locus if there was a proportion of <0.75 reads per allele. We checked that the dyadic likelihood of relatedness did not exceed 0.4 between any individual within zone A and zone AB or zone B and zone AB using the program COANCESTRY v1.0.1.2 (Wang 2011).

The output from STACKS consisted of 16,569 loci that we applied additional filtering steps to with a custom script (Myers, unpublished data) implemented in R STUDIO v1.0.136 (R Core Development Team 2008). Loci were removed if they appeared in the negative control and were observed in less than 85% of samples. We used a principal component analysis (PCA) in the R package *adegenet* v2.0.1 (Jombart 2008) to explore preliminary population structure. The putative clusters without admixed individuals were each analysed for loci out of Hardy-Weinberg Equilibrium (HWE) in the R package *pegas* v0.9 (Paradis 2010). We removed loci from further analysis that did not conform to HWE in (1) both putative clusters or (2) one putative cluster when they were only analysed in one cluster. We identified linked loci in each putative cluster excluding potentially admixed individuals, using PLINK v1.07 (Purcell *et al.* 2007). We removed loci from further analysis that were highly correlated ($r^2 >$ 0.1) in (1) both putative clusters or (2) one putative cluster when they were only analysed in one cluster. Within a linkage pair, we removed the locus with the most linkage pairs. When both loci had even numbers of linkage pairs, we removed the locus with the most missing data.

Differences between genetic clusters

F_{ST} outlier loci between putatively non-admixed individuals in zone A and zone B were identified using two programs. We ran BAYESCAN v2.1 (Foll and Gaggiotti 2008) with default settings after data format conversion with PGDSPIDER v2.1.1.0 (Lischer and Excoffier 2012) and the R package *OutFLANK* v0.1 (Whitlock and Lotterhos 2015). F_{ST}

outlier loci were defined as having a *q*-value and corresponding false discovery rate of < 0.1. Using a concensus list of F_{ST} outlier loci from both analyses, the dataset was separated into two versions, one with neutral loci (n-SNP), and one with outlier loci putatively under selection (o-SNP). The closest known species relative with an available whole genome sequence is the zebra finch (*Taeniopygia guttata*). We performed a discontinuous megablast search that looked for sequence similarities between TBGW o-SNPs and the zebra finch genome. To further understand divergence between the populations, we performed an analysis of molecular variance (AMOVA) and calculated the significance of pairwise F_{ST} between zones using GENODIVE v2.0b27 (Meirmans and Van Tienderen 2004) with 10,000 permutations. We tested differences between genetic clusters in two separate analyses; one where the region of parapatry was merged with zone A and another where the region of parapatry was merged with zone B. This was to determine how the variance and divergence between genetic clusters is affected by potential gene flow. We repeated these analyses with both n-SNP and o-SNP datasets.

Population structure and admixture

We tested for Isolation-By-Distance (IBD) by calculating geographic and genetic distance matrices for eleven localities that excluded the site MTB as it contained only one individual (Figure 4.1). The euclidean distance between localities (km) was first calculated in GENALEX v6.5 (Peakall and Smouse 2012; Peakall and Smouse 2006), which was then corrected to avoid passing through a salt lake, and were log transformed to account for individuals moving in two dimensions. We calculated a pairwise F_{ST} genetic distance matrix with n-SNPs using GENODIVE and also transformed the genetic data ($F_{ST}/1 - F_{ST}$) (Nei 1977). Tests for IBD are biased by hierarchical population structure where allele frequencies are sharply divided geographically (Meirmans 2012) and apriori evidence suggests that the

TBGW subspecies studied here have considerable population structure (Slender *et al.* 2017; Chapter 2). To determine whether IBD may be creating substructure within the subspecies, we performed a series of Mantel tests: (1) across locations within zone A (*A. m. indulkanna*), (2) across locations within zone B (*A. m. raglessi*), (3) across locations within zone A and zone AB, and (4) across locations within zone B and zone AB. We included zone AB (the region of parapatry) in an analysis with zones of both subspecies because individuals in this region are likely to belong to either or both genetic clusters (Slender *et al.* 2017; Chapter 2). Where IBD was detected, we also performed a partial Mantel test to assess whether the correlation between geographic and genetic distance is affected by population structure within the region of parapatry. We used a binary matrix that distinguished between comparisons of locations made either between or within zones (Drummond and Hamilton 2007). We used GENODIVE to compare matrices with 1000 permutations.

We used spatial autocorrelation (Smouse and Peakall 1999) in GenAlEx v6.5 (Peakall and Smouse 2006; Peakall and Smouse 2012) to further evaluate spatial structure in the genetic data at an individual level. A pairwise matrix with Roussets's *a* (Rousset 1997; 2000) genetic distance between all individuals with the n-SNP dataset was calculated using SPAGeDi v1.4b (Hardy and Vekemans 2002). Geographic distances between individuals were calculated in GenAlex v6.5 using the same method to create the geographic distance matrix described for the above IBD analysis. Distance classes were sufficiently small enough to evaluate any non-linear correlations with the autocorrelation coefficient (*r*) and where the sample size within each distance class was relatively even. We looked for the presence of IBD within each distance class as well as the detectibility of IBD across mulitple distance classes (Diniz-Filho and Pires de Campos Telles 2002). Significance was assessed for both tests using 95% confidence intervals for the null hypothesis of no spatial structure using 999 random

permutations, and for estimates of *r* by bootstrapping 999 pairwise comparisons for each distance class.

We investigated population structure and admixture using the n-SNP dataset with two methods: (1) Discriminant Analysis of Principal Components (DAPC) (Jombart *et al.* 2010) implemented in the R package *adegenet* v2.0.1 (Jombart 2008), which infers genetic clusters of individuals that are most closely related; (2) Bayesian clustering with the program STRUCTURE v2.3.4 (Falush *et al.* 2003; Pritchard *et al.* 2000) that determines genetic clustering based on HWE. Both methods are useful for detecting admixed individuals. For the DAPC, we retained one principal component, as this returned the optimum *a*-score, which is the difference between the proportions of successfully reassigned individuals compared to the number of principal components retained. The optimum number for *K* was inferred from the retained principal components by identifying *K* where the Bayesian Information Criterion (BIC) produced an elbow in the curve of BIC values as a function of *K*. Admixture was inferred if the proportion of population assignment was <0.9 or >0.1 in any individual. For the STRUCTURE analysis, three replicate runs for each *K* were analysed using default settings, unless stated otherwise. We used the admixture model with correlated allele frequencies and an MCMC chain of 1,000,000 iterations with a burnin of 10,000 iterations to test *K* between 1 and 5. To estimate the probability of mixed ancestry for each individual, the option ANCESTDIST was used. Admixture was inferred if the confidence intervals of the individual population assignment did not include 1 or 0 in all three replicate runs. STRUCTURE HARVESTER (Earl and vonHoldt 2011) was used to estimate the best fitting value for *K*. When the highest LnP(*K*) was not $K = 1$, then the most likely *K* was determined using Delta *K* (Evanno *et al.* 2005). Cluster assignments across three replicate runs of *K* were merged in CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007) and results were visualized

with DISTRUCT v1.1 (Rosenberg 2004). Further hierarchical population structure was investigated by repeating the analysis on individual populations detected in the initial run.

In areas where TBGWs were present, the two TBGW subspecies that are the focus of this study were correlated with the proportion of cover from different plant communities in areas of their core distribution (Chapter 3). We previously identified three principal components associated with these different plant communities (Chapter 3) and used latent factor mixed models (LFMMs) to test for associations between genotype defined by the combination of o-SNP and n-SNP datasets and the environmental variables defined by the principal components (Frichot *et al.* 2013). The LFMM test was performed in the R package *lfmm* v0.0.

We tested the proportion of migration between the three zones with a reduced dataset of 200 loci in BAYESASS v3.0.4 (Wilson and Rannala 2003). The 200 loci with the highest loading were chosen for the analysis by performing a PCA in the R package *adegenet* v2.0.1 (Jombart 2008) using the n-SNP dataset that included all individuals. Three independent MCMC runs were performed with 1,000,000 iterations and a burn-in of 10,000 iterations. The alpha (allele frequency) and delta (inbreeding coefficient) values were adjusted to 0.6 and 0.4 respectively so that the acceptance rates were between 20% and 60%. Iterations were sampled every 100 intervals to determine the posterior distribution of the parameters. Convergence of the MCMC run was assessed by inspecting the trace file in the program TRACER v1.6.0.

Results

We used samples from across three zones: zone A (n = 44), zone B (n = 61), and zone AB (n = 13) to assess gene flow between two parapatric subspecies. Following DNA extractions, FTA samples produced considerably lower quantities of DNA (<500 ng) compared to blood stored in salt solution (>1,000 ng). Following illumina sequencing, the average number of

reads per sample (before filtering) was 2,539,005 with a coefficient of variation of 24.6%. The between run reproducibility, calculated when there was a call for the same locus in duplicates on different plates, was 95.7% (n = 12,192). The average within run reproducibility, calculated when there was a call for the same locus in duplicates on the same plate, was 90.5% (n = 146,304). The genotyping error rate, calculated from the average number of allelic mismatches across duplicates, was 0.31% (n = 316,992). There were no individuals that exceeded > 30% missing data and in total, the dataset contained 5.56% missing data. Following SNP calling in STACKS, we removed 5 loci that appeared in the negative control and 2929 loci that had low coverage across samples. An initial PCA showed two putative genetic clusters with individuals from zone A forming one cluster, individuals from zone B forming the second cluster and 19 potentially admixed individuals from zone AB and zone B (Figure S 4.1). We observed similar amounts of missing data between the clusters, excluding potentially admixed individuals (cluster 1 [zone A]: 6.41%, cluster 2 [zone B]: 6.58%). We removed a further 625 loci from further analysis that did not conform to HWE and 5428 loci that were likely to introduce linkage disequilibrium.

We analysed a total of 7582 nuclear loci in the following analyses. Estimated mean heterozygosity (H) across all SNP loci per sample varied from 0.234 (± 0.003) in a sample from zone A to 0.301 (± 0.003) in samples from both zone A and zone B. Mean estimates of H were similar between the zones (zone A and zone B = 0.279 ± 0.002; zone AB = 0.270 ± 0.003). We identified 39 loci as potential F_{ST} outliers under selection (o-SNP) and the remaining 7543 loci were treated as neutral loci not under selection (n-SNP). Only four TBGW loci had similar sequence matches to the zebra finch genome (Table S 4.1). Zone B had slightly more polymorphic loci in the n-SNP dataset (99.9%) compared to zone A (99.2%). Using the n-SNP dataset, the proportion of total genetic variance explained by geographic population (zone A and zone B) did not change when the region of parapatry

(zone AB) was combined with either zone A or zone B (Table 4.1). Using the o-SNP dataset, the proportion of total genetic variance explained by population (zone A and zone B) was greater when the region of papapatry (zone AB) was combined with zone B (0.19%, p <0.001) compared to when zone AB was combined with zone A (0.15%, p <0.001; Table 4.1). Using the n-SNP dataset, there was no difference in pairwise estimates of F_{ST} when the region of parapatry was combined with either zone A or zone B (Table 4.2). The pairwise estimates of F_{ST} using o-SNP was higher when zone AB was combined with zone B (0.202, p <0.001) compared to when zone AB was combined with zone A (0.165, p <0.001; Table 4.2).

IBD was only detected in one of four Mantel tests that were performed (Figure S 4.2). There was a correlation between genetic and geographic distance across localities in zone A and zone AB (R^2 = 0.112, Rxy = 0.335, p = 0.029). There was no correclation between genetic and geographic distance across localities in zone B and zone AB (R^2 = 0.012, Rxy = 0.110, p = 0.435), zone B alone (R^2 = 0.036, Rxy = 0.190, p = 0.669), or zone A alone (R^2 = 0.084, Rxy = 0.290, p = 0.075). Using a partial Mantel test, the correlation between genetic and geographic distance across localities in zone A and zone AB was nearly significant (Rxy = 0.304, p = 0.064) when accounting for possible population structure between localities in zone A and zone AB. A larger sample size may improve the association between geographic and genetic distance for the partial mantel test. At the individual level there was positive spatial autocorrelation for the first six distance classes (0-20 to 100-120 kms) as well as the eigth and tenth distance classes (140-160, 180-200 kms) (Figure 4.2). When plotting r as a function of increasing distance classes, the curve intercepted the x-axis at 123.6 kms (Figure 4.2). IBD was detectible from 0 - 60 km and between 80-100 and 140-160 km (p < 0.01). This suggests that spatial autocorrelation is linear up to 60 kms and non-linear at other intervals, which may be caused by a heterogeneous landscape.

The result from STRUCTURE identified two major genetic clusters (Table S 4.2) corresponding to eastern and western populations. Two genetic clusters were also identified by the DAPC analysis albeit with weaker support (Figure S 4.3). Using $K = 2$, results from both STRUCTURE and DAPC were concordant in that both analysis showed that 1) zone AB contained the highest proportion of admixed individuals 2) there were greater proportions of admixture in individuals in zone AB than either zone A or zone B, and 3) there were greater proportions of admixture in individuals in zone B than zone A (Figure 4.3). Comparison of the two methods showed there were discrepancies in the identity of admixed individuals as well as in the proportions of admixture. The DAPC method compromises the power for detecting admixture with assigning individuals to populations, therefore we have limited the discussion of admixture below to the STRUCTURE results. In zone A, 2.3% of individuals were admixed and these individuals had a relatively low proportion of assignment probability from the eastern genetic cluster (< 18%). In zone B, 18% of individuals were admixed and these individuals had low to high proportions of assignment probability from the western genetic cluster (18.7 – 52.0%). Two of the admixed individuals in zone B came from museum samples that were collected in 1985 or 2007 and were from localities furthest from the region of parapatry (MUR and MTL). In zone AB, all individuals were admixed and had low to high levels assignment probability from both the eastern (17.5 – 71.4%) and western genetic clusters (28.6 – 82.5%). To look at hierarchical substructure within the identified populations, individuals in zone B and then zone A were excluded from two separate STRUCTURE analyses; for zone A and zone AB, $K = 1$ was most likely using the mean LnP(K) and for zone B and zone AB, $K = 3$ was most likely using Delta K (Figure S 4.4). Zone B comprised of two groups of individuals that were likely to be distantly related and formed two of the clusters in the STRUCTURE analysis of zone B and zone AB. An earlier analysis with COANCESTRY showed that the Dyadic likelihood and the 95% confidence

intervals for those groups were: r = 0.28 (0.26,0.30) – 0.30 (0.28,0.32) for three individuals in the first cluster and r = 0.14 (0.12,0.16) – 0.28 (0.25,0.30) for six individuals in the second cluster.

Using K = 2, the LFMM analysis identified 328, 333 and 419 loci associated with the vegetation variables PC1 (*Atriplex vesicaria* and *Zygochloa paradoxa*), PC2 (*Maireana* spp) and PC3 (*A. nummunlaria omissa*, *Rhagodia spinescens* and *Acacia* spp) respectively. Of the F_{ST} outliers, there were 12 loci that correlated with PC2 (two of these also correlated with PC3) and, in total, six loci that correlated with PC3. The results from BAYESASS suggested that zone AB received the more migrants per generation than zone A or zone B (Figure 4.4). There was more migration from zone A into zone AB than from zone B; the mean ± SD migration from zone A = 21.0 ± 4.7% and from zone B = 10.3 ± 4.6%. There was some migration per generation into zone B from zone A (4.5 ± 1.6 %), but there was < 1% migration per generation from either zone B or zone AB into zone A.

Discussion

We found that admixture between two TBGW subspecies was greatest within a region of parapatry and that gene flow strength varied according to the complexity of the landscape. Levels of inter-subspecific geneflow were greatest in the region of parapatry (zone AB) where there was a mixed ecotype (Chapter 3). In the core areas for *A. m. raglessi* (zone B) and *A. m. indulkanna* (zone A), grasswrens were predominantly present in a single ecotype (Chapter 3) where inter-subspecific gene flow was low. The mix of ecotypes in the region of parapatry included two ecotypes that were dominant in either the core area for *A. m. indulkanna* (zone A) or *A. m. raglessi* (zone B) and an ecotype found on a dune field separating the region of parapatry from the core area for *A. m. indulkanna* (zone A). There were a number of outlier loci associated with the ecotypes found in the core areas of the

either subspecies. Inter-subspecific gene flow was concordant with the distribution pattern of two mtDNA lineages, which showed asymmetric introgression from west to east (Chapter 2). There was considerable introgression of mtDNA haplotypes that suggest the two parapatric TBGW subspecies are undergoing divergence with gene flow and that ecological heterogeniety with varying levels of landscape complexity may facilitate divergence with gene flow between these subspecies.

Strong gene flow between TBGW subspecies is predominantly associated with high ecological heterogeneity. This study shows that high gene flow between *A. m. raglessi* and *A. m. indulkanna* mostly occurred in an area restricted by salt lakes to the north and south and that contained patches of different habitat types. Patches of one habitat type was likely to be unsuitable for either subspecies and two other habitat types were suitable for either one of the subspecies (Chapter 3). A non-linear signal of spatial autocorrelation above 60 km also suggests that gene flow may be affected by different dispersal distances associated with landscape heterogeneity. We did not test levels of ecological heterogeneity outside the region of parapatry because the small spatial scale needed to identify dominant ecotypes in arid Australian landscapes (see Tongway and Ludwig 1994) precluded measuring ecotype heterogeneity over a large area (Chapter 3). Greater habitat heterogeneity can facilitate local adaptation when there is moderate to strong selection because greater dispersal across habitat patches can increase genetic variance across the landscape (Forester *et al.* 2016).

Weak gene flow in the core areas of the TBGW subspecies could be associated with selection against particular phenotypes in areas with different ecotypes This is supported by the finding that 1) the habitat was only suitable for one or the other of the subspecies in each of the core ares of the subspecies (Chapter 3), 2) there were a number of outlier loci associated with the core areas of the subspecies, 3) a proportion of the outlier loci were associated with particular

habitat types, and 4) morphological divergence was greatest between individuals in the core areas of the subspecies (Slender *et al.* 2017; Chapter 2). Low levels of nuclear inter-subspecific gene flow in the core area for *A. m. raglessi* (zone B) was not enough to disrupt patterns of morphological differentiation and could also be the reason why mtDNA paraphyly was detected in *A. m. raglessi* (Slender *et al.* 2017; Chapter 2). We suggest that a reproductive barrier between the two subspecies does not currently exist because there is nuclear and mtDNA genetic discordance in the core area for *A. m. raglessi* (e.g. Toews and Brelsford 2012). Within the region of parapatry, we suggest that hybrid fitness was not affected by selection for particular phenotypes because there was a mixed ecotype and hybrid fitness can be greatly dependent on environment (Arnegard *et al.* 2014).

Distinguishing between divergence with gene flow and divergence in allopatry with secondary contact is difficult because these processes create similar patterns of divergence (Cruickshank and Hahn 2014). We suggest there is gene flow between the two divergent TBGW subspecies but do not exclude the possibility that the subspecies may have been allopatric in the past. The non-overlapping distributions of the TBGW subspecies are concordant with contemporary patterns in the landscape that appear to be preventing nuclear introgression (Chapter 2; Chapter 3). Concurrence between ecotype and patterns of divergence have been used to distinguish been evolutionary processes in other species (Butlin *et al.* 2014; Galligan *et al.* 2012). Estimates place the time of most recent common ancestor for *A. m. indulkanna* and *A. m. raglessi* for the mtDNA ND2 gene at ~400 kya (Austin *et al*. 2013) or ~750 kya (Norman and Christidis 2016) depending on how the substitution rate was calibrated. This could be associated with the emergence of different plant communities (Norman and Christidis 2016) or allopatry in isolated refugia caused by an expansion of the arid zone during the Pleistocene (Byrne *et al*. 2008). Introgression of western mitochondrial haplotypes into the nuclear genetic background of *A. m. raglessi* may have occurred

following secondary contact. This is likely to have taken place subsequent to the last glacial maximum (21, 000 ± 2000 years ago) when extensive areas of active dune fields became stable through the growth of modern floral communities (Byrne *et al*. 2008). Further work is needed to investigate the demographic history of the subspecies and determine how previous allopatric distributions may have affected subspecies divergence.

TBGW subspecies show asymmetric geneflow from *A. m. indulkanna* to *A. m. raglessi*. Low levels of gene flow were permitted from *A. m. indulkanna* to *A. m. raglessi* within the core area for *A. m. raglessi* (zone B) but we did not detect gene flow from *A. m. raglessi* to *A. m. indulkanna* within the core area for *A. m. indulkanna* (zone A). Within the region of parapatry, there does not appear to be a predominant subspecies lineage as individuals were highly admixed and there were relatively even proportions of each mitochondrial haplogroup. The dune field that runs between Lake Eyre and Lake Torrens demarcates the boundary of the asymmetry. Individuals with a genetic background of *A. m. raglessi* occur only on one side of the dunefield (east) whereas individuals with a genetic background of *A. m. indulkanna* occur on both sides. Asymmetric gene flow could be due to a number of processes. These include greater niche breadth in *A. m. indulkanna* (Chapter 3), demographic or ecological differences on either side of the dune field (e.g. Oswald *et al.* 2017), or a mating advantage for *A. m. indulkanna* (e.g. Baldassarre and Webster 2013; Baldassarre *et al.* 2014). We did not detect any individuals with an eastern haplotype and a predominant nuclear genetic background of *A. m. indulkanna.* Mito-nuclear incompatibilities in the *A. m. indulkanna* nuclear genetic background may be associated with partial reproductive isolation or ecological adaptation for a particular mtDNA genome in the ecotype west of the dune field (Hill 2015; Morales *et al.* 2015). Further work is needed to test hypotheses regarding subspecies behaviour (see Chapter 5) and habitat preference, landscape ecological productivity and stability, and mitochondrial incompatibilities.

This study shows that two parapatric TBGW subspecies have high gene flow in a region of parapatry that contains heterogeneous ecotype patches and signals of selection between the core areas of the subspecies that contain particular ecotypes and where gene flow is low. These landscape features are likely to affect dispersal distances, the location of the subspecies parapatric boundaries and may be associated with patterns of asymmetric gene flow from *A. m. indulkanna* to *A. m. raglessi.* Accounts where gene flow is likely to contribute to patterns of divergence are increasing (e.g. Rollins *et al.* 2012; Baldassarre *et al.* 2014; Butlin *et al.* 2014; Grant and Grant 2016; McLean *et al.* 2017; Peters *et al.* 2017). For subspecies of the TBGW, divergence with gene flow is affected considerably by the geographic landscape. These findings enhance our understanding of patterns of divergence between parapatric subspecies.

Acknowledgements

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Table 4.1 Partitioning of the molecular variance among (1) individuals within zone A and zone B and (2) between zone A and zone B using AMOVA. Zone AB was merged with zone A and zone B in two separate analysis and variance compared for both n-SNP and o-SNP datasets. Significant *p*-values (<0.05) are shown in bold.

Zone combined with zone AB	Dataset	Source of variation	Nested in	Variance (%)	df	SS	F-value	*p*-value
A	n-SNP	Among individuals	Population	0.081	116	145109.166	0.082	**<0.001**
		Among populations	--	0.007	1	2197.339	0.007	**<0.001**
A	o-SNP	Among individuals	Population	0.105	116	806.051	0.124	**<0.001**
		Among populations	--	0.150	1	135.610	0.150	**<0.001**
B	n-SNP	Among individuals	Population	0.082	116	145111.592	0.082	**<0.001**
		Among populations	--	0.007	1	2194.607	0.007	**<0.001**
B	o-SNP	Among individuals	Population	0.094	116	788.943	0.115	**<0.001**
		Among populations	--	0.185	1	159.104	0.185	**<0.001**

Table 4.2 Pairwise differentiation when zone AB is merged with zone A or zone B for both the n-SNP and o-SNP datasets. Cells show F_{ST} and *p*-values in parentheses. *P*-values were calculated after 10,000 permutations. Significant *p*-values (<0.05) are shown in bold.

Zone combined with zone AB	n-SNP	n-SNP	o-SNP	o-SNP
	A	B	A	B
B	0.008 **(<0.001)**	--	0.202 **(<0.001)**	--
A	--	0.008 **(<0.001)**	--	0.165 **(<0.001)**

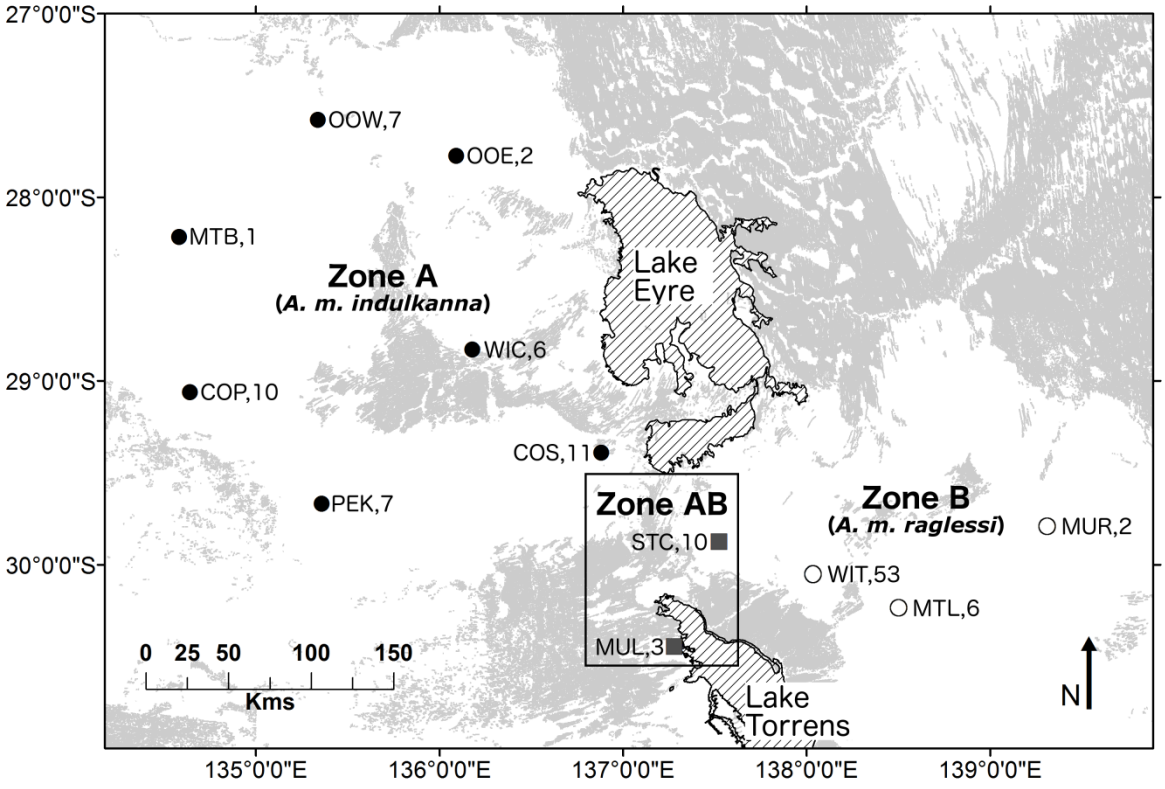

Figure 4.1 South Australian collection localities for samples of two thick-billed grasswren subspecies used for nuclear genetic sequencing. Localities are grouped into three zones according to their orientation to the sand dunes (grey shade) that run between Lake Eyre and Lake Torrens; zone A (solid circle) occurs to the west of the sand dunes, zone AB (grey square) occurs immediately east of the sand dunes in a region of parapatry, and zone B occurs to the east of zone AB. Locality abbreviations in map correspond to location codes listed in Appendix. Numbers indicate sample size at that locality.

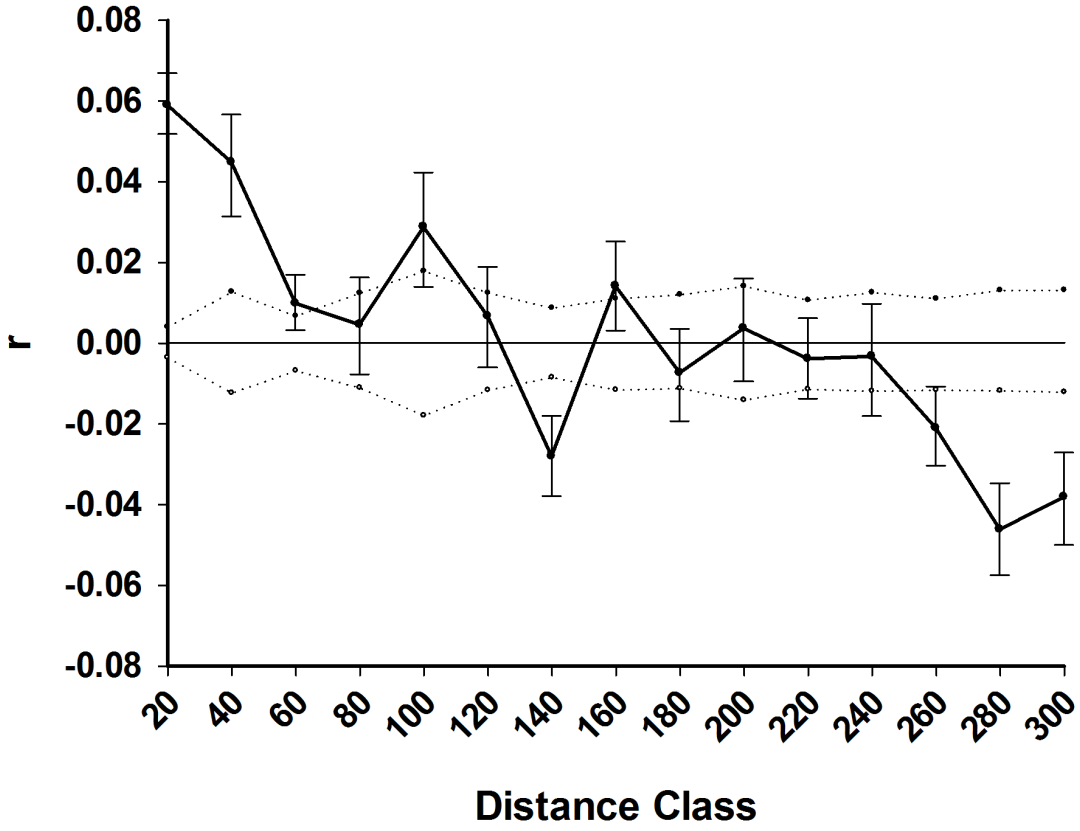

Figure 4.2 Correlogram showing the spatial autocorrelation coefficient *r* as a function of distance (km) indicated by distance class (end point). Dotted lines are the 95% CI about the null hypothesis of a random distribution of genotypes and error bars are 95% CI of *r*.

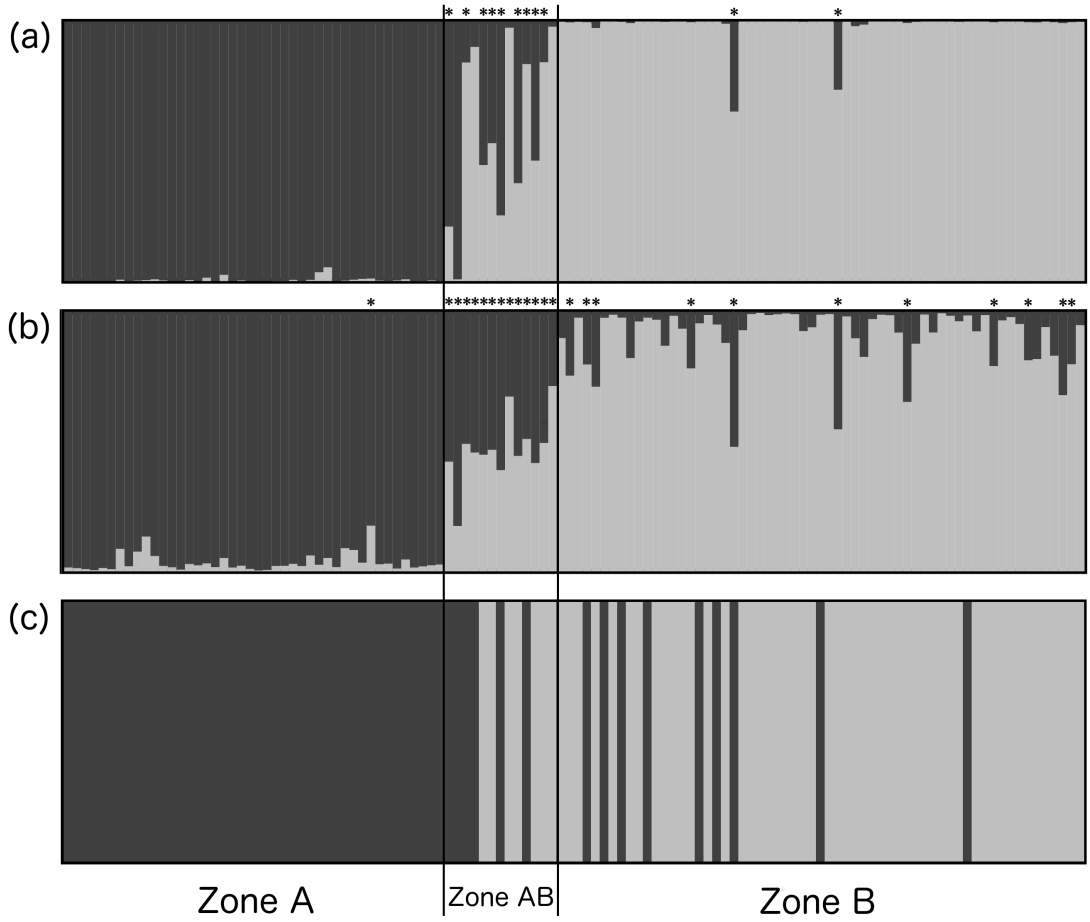

Figure 4.3 Population assignment tests using 7543 n-SNP loci where $K = 2$ for (a) DAPC and (b) STRUCTURE or using (c) mitochondrial haplotype for ND2 across three zones (Figure 4.1). Individuals are ordered by latitude in the order listed in the Appendix. The proportion of each colour shows the posterior mean proportion of ancestry from the subspecies *A. m. indulkanna* or western haplotype (dark grey) and *A. m. raglessi* or eastern haplotype (light grey). Individuals marked with an asterisk were identified as admixed.

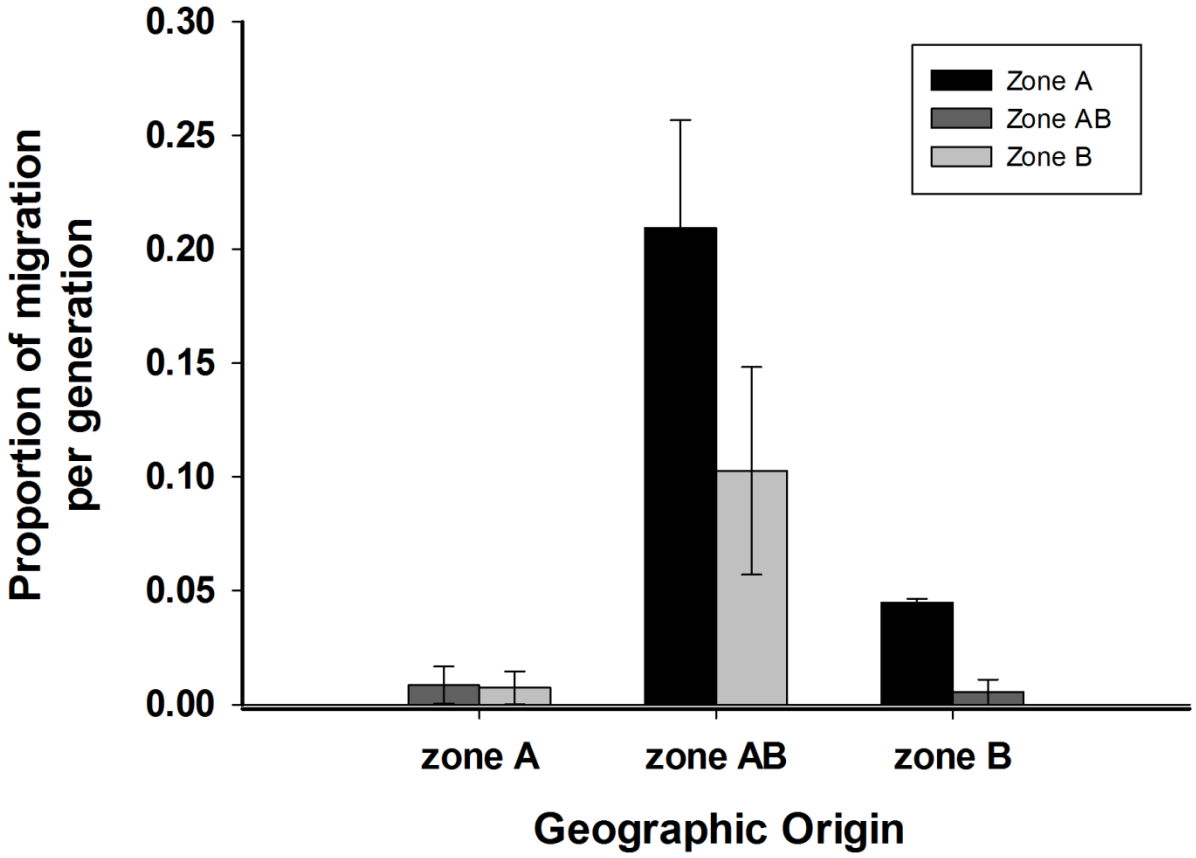

Figure 4.4 The proportion of migration (average and standard deviation) assessed between each zone (zone A, zone B, and zone AB) with BAYESASS. Migration from zone A is in black, migration from zone B is in light gray, and migration from zone AB is in dark gray. The analysis was performed using 200 loci that were identified following a PCA to determine the loci with the highest loading.

Table S 4.1 Results of discontinuous megablast for sequence similarity between outlier loci and the zebra finch (*Taeniopygia guttata*) genome. E – value is the expected number of hits at random, PI is the percent identity, PREDICTED means that the protein translations from the zebra finch sequences have not been tested experimentally but are only predicted to produce these proteins based on sequence similarity to other organisms.

Locus	Allele	Description	E-value	PI	Accession	Hit start	Hit end
84800	0	PREDICTED: *Taeniopygia guttata* multiple EGF-like-domains 10 (MEGF10), mRNA	8E-18	95	XM_002188758.1	1870	1925
	1	PREDICTED: *Taeniopygia guttata* multiple EGF-like-domains 10 (MEGF10), mRNA	2E-19	96	XM_002188758.1	1870	1925
47743	0	PREDICTED: *Taeniopygia guttata* SRY (sex determining region Y)-box 1 (SOX1), mRNA	1E-22	97	XM_002191013.3	359	298
	1	PREDICTED: *Taeniopygia guttata* SRY (sex determining region Y)-box 1 (SOX1), mRNA	1E-22	97	XM_002191013.3	359	298
111006	0	PREDICTED: *Taeniopygia guttata* metastasis suppressor 1-like (MTSS1L), mRNA	6E-13	98	XM_012570005.1	676	635
	1	PREDICTED: *Taeniopygia guttata* metastasis suppressor 1-like (MTSS1L), mRNA	6E-13	98	XM_012570005.1	676	635
75123	0	*Taeniopygia guttata* BAC clone TGMCBa-46H12 from chromosome unknown, complete sequence	2E-07	80	AC188191.2	112202	112144

1	*Taeniopygia guttata* BAC clone TGMCBa-49H12 from chromosome unknown, complete sequence	3E-17	90	AC199447.2	40426	40365

Table S 4.2 Results from STRUCTURE HARVESTER. When the highest LnP(*K*) (BOLD) is not *K* = 1, the highest Delta *K* is used to determine *K* (BOLD).

K	Reps	Mean LnP(*K*)	Stdev LnP(*K*)	Delta *K*
1	3	-800038.30	5.345	-
2	3	-798361.90	8.141	**147.695**
3	3	**-797887.83**	**41.600**	76.928
4	3	-800613.97	2253.739	0.410
5	3	-802417.10	2275.655	-

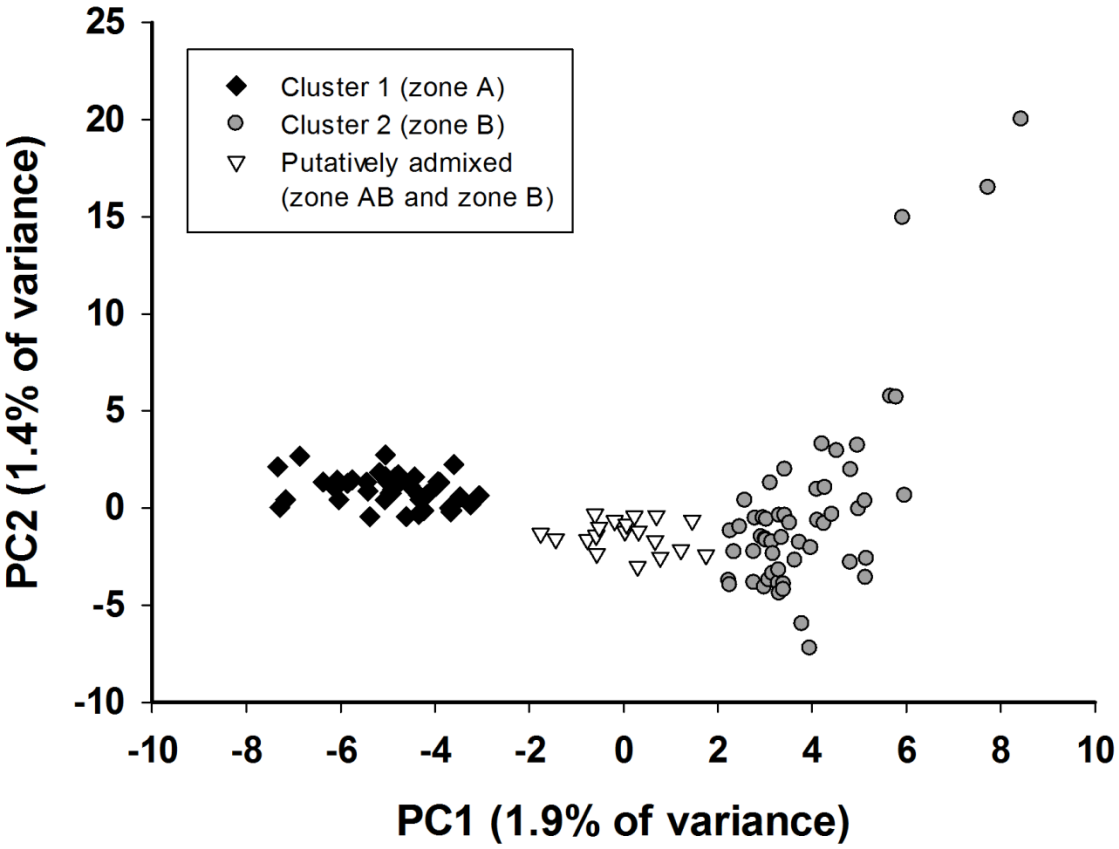

Figure S 4.1 Initial PCA with 13,635 loci used to identify putatively admixed individuals.

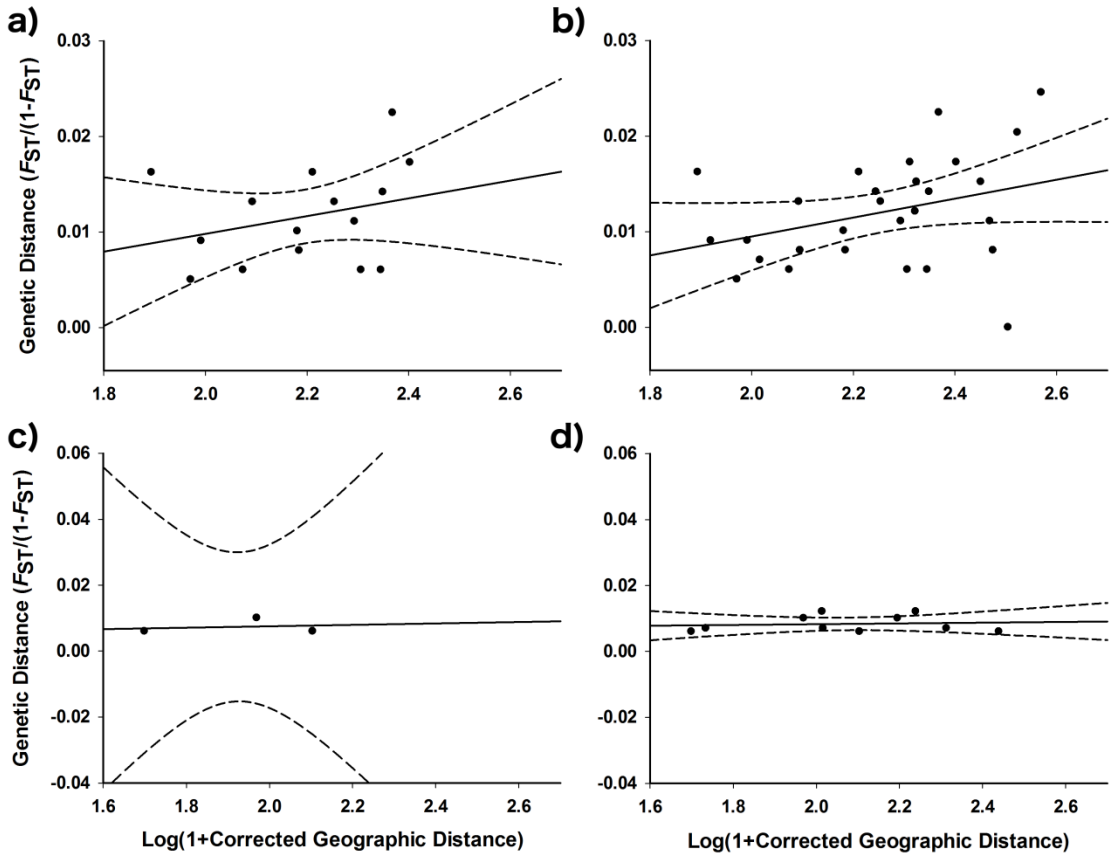

Figure S 4.2 The pairwise genetic ($F_{ST}/(1 - F_{ST})$) and geographic (log(1 + km)) relationship between localities by zone (zone A: $n = 6$, zone B: $n = 3$, and zone AB: $n = 2$) using a Mantel test. There was only one sample collected at the locality MTB, therefore this locality was excluded. The solid line is the line of best fit and the broken lines are the 95% confidence intervals. Different graphs represent comparisons of localities from different zones; a) zone A only, $R^2 = 0.084$, b) zone A and zone AB, $R^2 = 0.112$, c) zone B only, $R^2 = 0.036$, and d) zone B and zone AB, $R^2 = 0.012$.

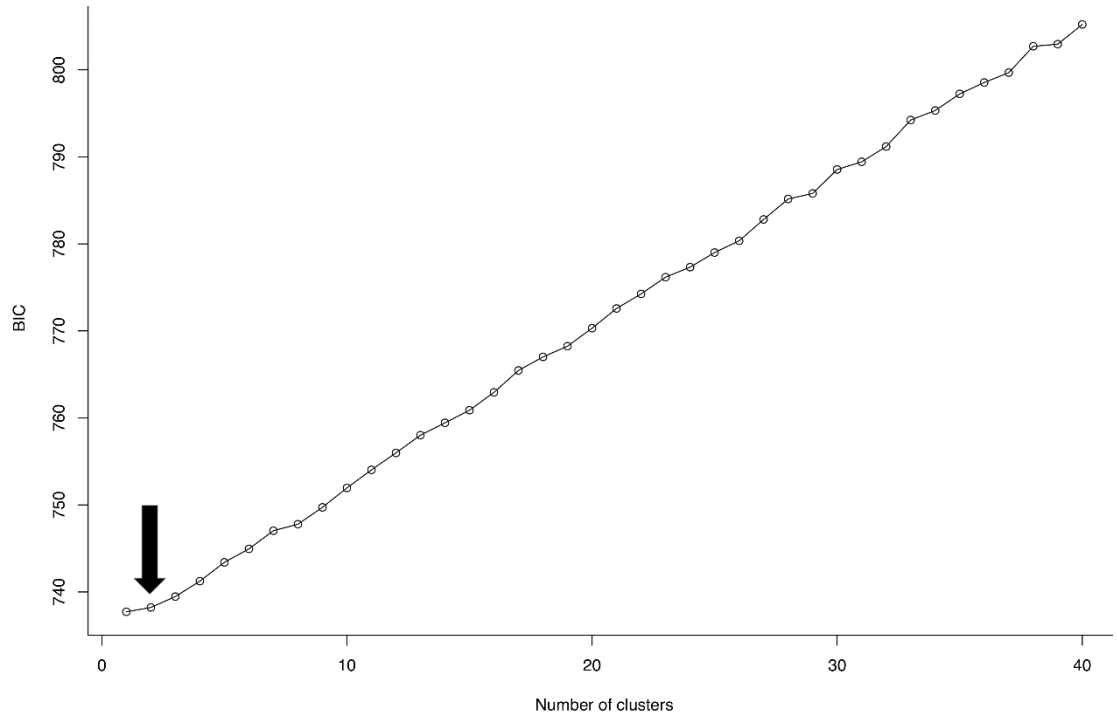

Figure S 4.3 Results from the DAPC analysis. *K* is inferred from the lowest BIC value that falls after an elbow in the curve of BIC values as a function of *K* (arrow).

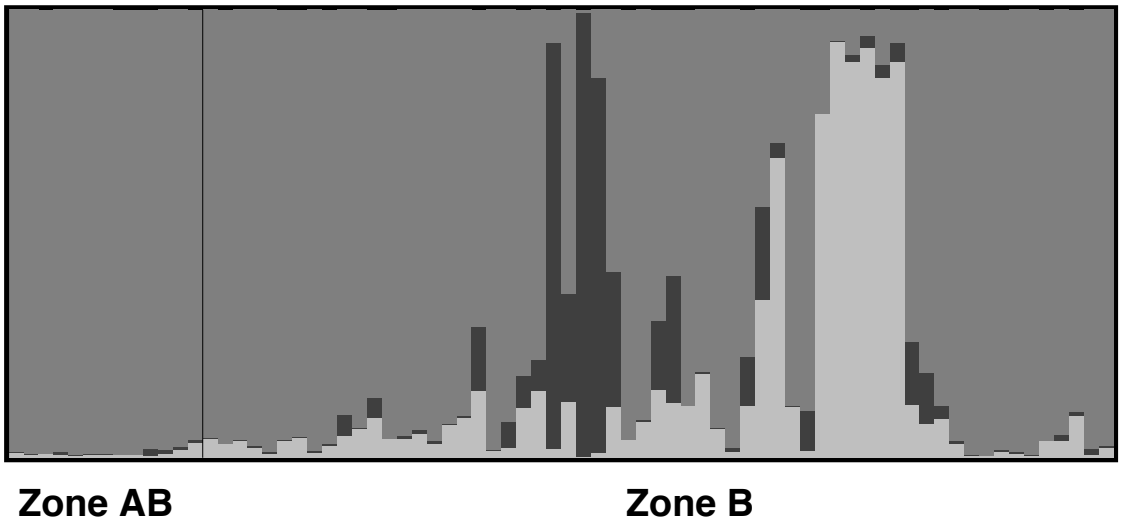

Figure S 4.4 STRUCTURE results with only individuals from zone B and zone AB (*n* = 74). Delta *K* showed the most likely *K* = 3. The two small clusters within zone B are likely to belong to family groups.

Chapter 5 Thick-billed grasswren (*Amytornis modestus*) song and behavioural response to song

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Abstract

Passerine song has many functions including mate attraction and territory defence. When songs across populations diverge, this can lead to changes in conspecific recognition and barriers to gene flow, which affect evolutionary processes that could lead to speciation. Two subspecies of thick-billed grasswren (*Amytornis modestus*) have a parapatric distribution characterised as a narrow region of high genetic admixture where the two subspecies meet. Outside the region of parapatry, the subspecies are genetically and morphologically diverged and weak inter-subspecific gene flow is asymmetric from *A. m. indulkanna* to *A. m. raglessi.* We examined the differences between song of *A. m. indulkanna* and *A. m. raglessi* and experimentally broadcast each subspecies song to compare territory-holder response in relation to intruder subspecies type. Our aim was to determine if different territorial responses to subspecies specific song could play a role in observed patterns of weak asymmetric genetic introgression. The song of each subspecies contained unique vocal elements that were absent in the other subspecies. *Amytornis modestus raglessi* responded similarly to con-subspecific and hetero-subspecific intruder song and *A. m. indulkanna* responded more often and with greater intensity to hetero- compared to con-subspecific intruder song. The stronger response by *A. m. indulkanna* towards hetero-subspecific

intruders reported here provides a behavioural explanation for the observed patterns of asymmetrical gene flow.

Introduction

Behavioural barriers to gene flow can affect rates of speciation and patterns of biodiversity (Coyne and Orr 2004; Price 2008). Many organisms use behavioural signals during mate choice that act as a pre-zygotic barrier to gene flow (Maroja *et al.* 2009). Hybrid zones are useful for studying how barriers to gene flow develop and are maintained when closely related populations are in contact, as this is a fundamental problem in biology (Punzalan and Rowe 2017). There are many cases where diverged populations that have made secondary contact across a hybrid zone show patterns of asymmetric introgression (Baldassarre *et al.* 2014; Beysard and Heckel 2014; Coyner *et al.* 2015). Asymmetric introgression can be explained by genetic (Borge *et al.* 2005; Devitt *et al.* 2011) or demographic processes (Belkacem *et al.* 2016; Johnson *et al.* 2015) but the importance of investigating behaviour as a possible explanation for asymmetric introgression has only recently been recognised (Toews and Brelsford 2012). Evidence is mounting for an association between mate choice and asymmetric gene flow (Beysard *et al.* 2015; Greig *et al.* 2015; Peters *et al.* 2017). Mate choice could be associated with asymmetric introgression because hybridising populations could respond differently to hetero-specific compared to con-specific individuals. Parapatric populations that show asymmetrical gene flow provide a unique opportunity to understand the role of behavioural barriers in preserving population divergence between geographically continuous populations.

In passerine birds, rates of song evolution have been shown to coincide with rates of speciation (Mason *et al.* 2017). Song evolution is important in diverging bird populations because birds use differences in song characteristics, such as frequency (kHz) or vocal

elements, for species recognition (Dingle *et al.* 2010). Between subspecies that have few differences in song, playback experiments are a better indicator of whether individuals can distinguish between subspecies than song analysis (Freeman and Montgomery 2017). It has been experimentally shown that songbirds are more likely to produce a territorial response to conspecific than heterospecific intruders (Catchpole and Slater 1995; Collins 2004; Price 2008). The explanation for such a pattern is that typically, a male defending his territory will respond to con-specific intruder song because the con-specific intruder is recognised as a sexual competitor (Christensen *et al.* 2009). The defending male does not usually respond strongly to hetero-specific intruder song because the hetero-specific is not recognised as a sexual competitor. However, hetero-specific intruders may pose a threat in other situations, for example if resources were limited or higher density increases predation risk. Clearly, a range of circumstances may shape complex interacting factors related to territorial behaviour (Baldassarre and Webster 2013; Ord and Stamps 2009; Stein and Uy 2006). Defining differences in territorial behaviour between closely related lineages is useful for understanding how song evolution leads to behavioural barriers to gene flow.

The Maluridae is a passerine family that are endemic to Australia and Papua New Guinea. It consists of three genera, the fairywrens (*Malurus*), emuwrens (*Stipiturus*) and grasswrens (*Amytornis*). Fairywrens have complex song and unusual singing behaviour. Individuals have multiple vocalisation types used for territorial defence and mate attraction and both males and females sing solo song to defend their territory (Cooney and Cockburn 1995; Greig and Webster 2014; Hall and Peters 2008; Rowley and Russell 1997). Territorial behaviours are important for fairywrens because these birds are sedentary, defend territories year-round, and breeding pairs have high rates of extra-pair copulations whereby paired females solicit extra-pair copulations from non-pair males (Brooker *et al.* 1990; Double and Cockburn 2000). Particular song traits are highly correlated with testes mass in a number of fairywren species,

suggesting that the evolution of song in these species is under sexual selection (Greig *et al.* 2013). Distinguishing between con-specific and hetero-specific intruders may be important for diverging populations of fairywrens. Superb fairywren (*Malurus cyaneus*) populations in South Australia have been shown to discriminate between hetero-subspecific and con-subspecific intruders using song (Kleindorfer *et al.* 2013). In the red-backed fairywren (*M. melanocephalus*), asymmetric morphological introgression between parapatric subspecies was correlated with an asymmetric response to hetero-subspecific song (Baldassarre *et al.* 2014; Greig *et al.* 2015). The Maluridae is arguably the best studied bird group in Australia in regards to behaviour and ecology (Buchanan and Cockburn 2013; Greig *et al.* 2013), and phylogeny (Christidis *et al.* 2010; Joseph *et al.* 2013). However, many species of grasswren are virtually unstudied and little is known about territorial behaviour in the *Amytornis* group. Comparing grasswren territorial responses to hetero- and con-specific intruders could reveal evolutionary processes related to divergence within the Maluridae.

Differences in territorial response to hetero- and con-subspecifics may be affecting patterns of inter-subspecific gene flow between two subspecies of the thick-billed grasswren (*Amytornis modestus*). This could influence subspecies divergence and/or risk of extinction. The subspecies, *A. m. indulkanna* and *A. m. raglessi* have a region of parapatry and are not currently reproductively isolated as there are high levels of inter-subspecific gene flow in this region (Chapter 4). There is no evidence for inter-subspecific gene flow within the core area for *A. m. indulkanna* but there were low levels of inter-subspecific gene flow and mito-nuclear discordance within the core area for *A. m. raglessi*. The subspecies *A. m. raglessi* is of conservation concern due to habitat loss from overgrazing by livestock (Louter 2016). *Amytornis modestus raglessi* is classified as ‘vulnerable’, and *A. m. indulkanna* is classified as ‘of least concern’ (Garnett *et al.* 2011). It is a conservation priority to understand drivers of asymmetric gene flow in this context because threatened populations may benefit from

increased genetic diversity as a consequence of unidirectional introgression (Hamilton and Miller 2016; Harrisson *et al.* 2016). Here, we ask if two thick-billed grasswren subspecies differ in their territorial response to the song of con-subspecific intruder birds to determine whether this could be a cause of the observed inter-subspecific gene flow asymmetry.

Using song recordings of *A. m. indulkanna* and *A. m. raglessi*, we performed playback experiments to measure the behavioural response of the territory holder to an intruder of the same or of a different subspecies. If the behavioural response of the territory holder predicts gene flow, then we expect that the population with intra-subspecific gene flow only, *A. m. indulkanna*, will discriminate between intruder birds from different subspecies. *Amytornis modestus raglessi* previously showed inter-subspecific gene flow and therefore should have a comparable response to intruder song from either subspecies. We hypothesise that subspecies differences in territorial response to intruder song will affect patterns of inter-subspecific gene flow.

Methods

Study species and study sites

Thick-billed grasswrens (*Amytornis modestus*, TBGW) are cursorial passerines that occur in the arid zone of South Australia, Northern Territory and New South Wales (Black *et al.* 2011). They are facultative cooperative breeders with large territories (Louter 2016) and have no documented cases of extra-pair paternity (Louter 2016). Females are distinguishable from males as they have a rufous patch on their flanks and males do not (Rowley and Russell 1997). Two TBGW subspecies (*A. m. indulkanna* and *A. m. raglessi*) have diverged to the east and west of the South Australian salt lakes, Lake Eyre and Lake Torrens (Slender *et al.* 2017; Chapter 2). They are currently parapatric and have a relatively narrow region of high

gene flow (Chapter 4). Data were collected from study sites outside the region of parapatry where divergence between the subspecies was greatest. Study sites occured within seven areas of approximately 2500 km^2. We sampled *A. m. indulkanna* from five areas to the west of Lake Eyre and Lake Torrens: 1) Cooper Pedy, 2) Coward Springs Railway Siding, 3) Oodnadatta, 4) Peculiar Knob and 5) William Creek (Table S 5.1). We sampled *A. m. raglessi* from two areas to the east of Lake Eyre and Lake Torrens: 1) Witchelina Nature Reserve and 2) Mount Lyndhurst station (Table S 5.1). We were unable to record song within the region of parapatry and sample sizes for playback experiments within the region of parapatry were too small to include in this study.

Song recordings

TBGW song was most often heard early in the morning and was often sung multiple times over a short period. Singing was usually observed for a single bird perched on top of a shrub most likely within an established territory. Grasswrens have multiple vocal types (Rowley and Russell 1997). TBGW song has a broad frequency bandwidth and consists of multiple element types including trills, buzzes, and up- and down- slurred notes (Figure 5.1). In this system, we considered a vocalisation to be a song if it had complex element composition (multiple element types) and had been produced during the breeding season. These features have been used to distinguish different vocalisation types in other grasswren species (Rowley and Russell 1997).

We used song from the zebra finch (*Taeniopygia guttata*) as a hetero-specific control and had a total of 50 stimuli made from recordings of TBGWs and zebra finches (Table S 5.1). The number of stimuli for each song group was as follows: *A. m. raglessi* = 20 (all recorded at Witchlina Nature Reserve), *A. m. indulkanna* = 18 (6 recorded at Coward Springs and 12 recorded at four other locations) and zebra finch = 12 (11 recorded at Millbrook and 1

recorded at Witchelina Nature Reserve). All TBGW stimuli and one zebra finch stimulus were recorded in the field during Aug – Sep of 2013 and 2014. The remaining 11 zebra finch stimuli were recorded from different captive individuals at the Rockefeller University Field Research Center, Millbrook, NY, USA.

Most recordings were made using a High Resolution Digital Audio Recorder 702 or 722 with a 48 kHz sampling rate and 24 bit-depth (Sound Devices, LLC, Reedsburg, WI, USA) connected to a NTG8 shotgun microphone (RODE Microphones, LLC, Long Beach, CA, USA; frequency response 0.04 – 20 kHz). Four recordings of *A. m. raglessi* were made using an H4n Handy Recorder, 44.1 kHz sampling rate and 16 bit-depth (Zoom Corporation, Hauppauge, NY, USA), at three different active nests. All recordings were later transformed to 44.1 kHz and 16 bit-depth. Excluding the recordings that were made at a nest, most recordings were opportunistically obtained between 0700 and 0930 or 1500 and 1730 (zebra finch = 1, *A. m. raglessi* = 10 and *A. m. indulkanna* =10) and the remaining were made during or following playback trials (*A. m. raglessi* = 6 and *A. m. indulkanna* = 8). For most grasswren recordings, we observed that a grasswren pair was present during the recording and most recordings were made during the nesting or fledgling phase (Table S 5.2). We noted that individual grasswrens sang songs that contained different combinations of vocal elements. To create the playback stimuli, we used songs containing different vocal elements for different playback stimulus even if those songs came from the same recording (Table S 5.1).

Recordings were edited in Audacity v2.0.3 (GNU General Public License v2.0). TBGW stimuli consisted of song lasting between 0.7 and 4.2 s (mean = 2.1 s) and contained between 3 and 13 different element types (mean = 7). This is comparable to what is reported in Rowley and Russell (1997), where the phrase length of most grasswren songs is approx. 2 s.

Both TBGW and zebra finch vocalisations were repeated at approximately 12 s intervals so that playbacks lasted for 3 min and consisted of five series of vocalisations per minute. Background noise was removed from stimuli by filtering below 1 – 3 kHz and above 10 – 15 kHz using Amadeus Pro v2.1.5 (HairerSoft, Kenilworth, UK). The amplitude of recordings varied across stimuli as the distance to grasswren, volume of the grasswren vocalisation and the environmental conditions greatly affected the recording quality. We amplified and normalised each stimulus to the peak amplitude of the song ensuring that songs were not clipped. The amplitude of the stimuli was tested using the decibel 10th App v5.1.2 (SkyPaw Co. Ltd), set at C-weighting and fast response. The average amplitude of stimuli was 81.4 dB SD ± 4.1. Playback quality was accounted for as a random effect in the experimental analysis (described below). All playbacks were performed with an Orbit MP3 iM237 speaker (Altec Lansing, Milford, PA, USA) with a frequency response 0.1 – 20 kHz and an iPod (Apple Inc., Cupertino, CA, USA).

Acoustical analyses

This is the first study that describes differences in song between subspecies of the TBGW. We performed a preliminary analysis to detect differences between TBGW subspecies song using all TBGW stimuli used for the playback experiments (this incorporated all available recordings of TBGW song made during this study where the song quality was reasonable). Spectrograms were created in Raven Pro v1.5 (Cornell Lab of Ornithology, Ithaca, NY, USA) for each song using the Hann algorithm (filter bandwidth 248 Hz, size 256 samples, time grid overlap 50%, grid resolution 2.90 ms, 172 Hz, DTF 256 samples). A library of different element types was created following visual inspection of similar and different elements in each spectrogram. In order to determine how many songs were needed to capture the element diversity within each subspecies, the number of new elements encountered was

graphed against the number of songs analysed producing an asymptotic curve. Unique subspecies elements were identified when an element type occurred in only one of the subspecies. We used a chi-squared analysis in SPSS Statistics v22.0 (IBM Corp., Armonk, NK, USA) to determine whether there was an association between subspecies and the number of unique elements.

Playback experiments

Playback trials were performed during the breeding season from Aug - Sep (Black *et al.* 2011) in 2014 and 2015 at 69 TBGW territories (*A. m. raglessi* = 40 and *A. m. indulkanna* = 29). We identified territories based on bird surveys performed in 2013 and 2014, where we found 1) a breeding pair or an active nest in any year or 2) multiple grasswren sightings at the same location within any year (Louter 2016; Slender *et al.* 2017; Chapter 2). On occasion (*n* = 16) we could not confirm whether individual grasswrens remained within the same territory across years. To control for pseudo-replication, we did not perform replicate playback experiments of the same treatment in a territory either within or between years. To minimise the likelihood that birds were familiar with the song treatment "own" (see below) prior to the playback experiment, we tested focal birds with songs that were recorded from non-neighbouring individuals.

A playback trial was only performed after the presence of a TBGW in a territory was confirmed by visual or auditory detection. TBGWs are often skittish and are easily flushed by an observer. We placed the speaker on the ground, concealed in vegetation, on average 44 m from the focal bird. The observer was concealed behind vegetation and sat on average 26 m away from the speaker. The set up of the speaker did not take longer than 5 min. We documented pre-playback and playback observations separately. Each playback experiment lasted for 3 min following 1 min of silence to perform pre-playback observations.

We tested the response to playback stimuli using different playback treatments per TBGW territory. The treatments were 1) own subspecies song, 2) other subspecies song and 3) control. In this study, we use the term "own" to describe any song from the same genetic subspecies as the focal bird regardless of population. Similarly, we use the term "other" to describe any song from a different genetic subspecies. This allowed us to compare the effects of divergence on song recognition. *Amytornis modestus indulkanna* received 16 own, 18 other and 20 control trials while *A. m. raglessi* received 24 own, 21 other and 23 control trials (Table S 5.3). In order to avoid pseudo-replication, the order of treatment types were randomly alternated across groups, and treatment order was also used as a random effect in the experimental analysis (described below). In some cases grasswrens were not present at a territory on an experimental day and therefore did not receive one of the treatments. To compare the behavioural responses of grasswrens to own versus other treatments, we recorded the following variables: 1) latency of the grasswren to approach within 20 m of the speaker, 2) latency of the grasswren to approach within 10 m of the speaker, 3) the minimum approach distance to the speaker, 4) the frequency that the grasswren was observed to cross the speaker and 5) the frequency that song was heard from the focal bird. A speaker cross was recorded when an individual grasswren approached and crossed over the speaker at ground level.

Statistical analyses of playback response

We performed two analyses: (1) to assess the proportion of trials where a territorial response was observed across different treatments (own, other and control) and (2) to assess territorial response intensity to different treatments (own and other) within the focal subspecies. We compared the proportion of response using a binomial response variable (0 or 1). A positive response was indicated by an approach to within 20 m of the playback, singing during

playback (where no song was heard during the pre-playback) and/or vigilance from further than 20 m. Where there was a positive binomial response (n = 47), we compared the intensity of the response to the two grasswren treatments (own and other). Because there is likely to be a correlation between the five response variables, we performed a principal component analysis (PCA) (Table 5.1) that generated an overall response intensity for the subset of trials where grasswrens responded. Response intensity was analysed using generalised linear mixed models (GLMMs) implemented in R studio v1.0.136 (RStudio, Boston, MA, USA) with the package lme4 v1.1 – 11 (Bates *et al.* 2015). We performed the GLMM analysis on both the reduced factor scores calculated with the PCA as well as on each univariate variable as PCA can mask responses when variables are combined. We controlled for stimulus ID (i.e quality), area of playback origin, territory ID and treatment order by including these variables as random effects in our analysis. To compare the proportion of response per playback treatment, we used mixed logistic regression models with binomial distributions and logit link functions. To compare the response intensity per playback treatment, we used linear-mixed models.

Results

Subspecies specific vocal elements

We created a preliminary library of vocal elements using 18 songs of *A. m. indulkanna* and 20 songs of *A. m. raglessi* (Figure 5.2). An asymptotic curve showed that element diversity was captured within 11 songs for *A. m. raglessi* and 8 songs for *A. m. indulkanna* (Figure S 5.1). We identified 32 vocal elements: 24 elements occurred in both subspecies (shared), 6 elements only occured in *A. m. raglessi* (unique) and 2 only occured in *A. m. indulkanna* (unique). There was no association between subspecies and the number of unique element types (likelihood ratio = 1.807, p = 0.179).

Proportion of response within subspecies

Of the 79 trials where a grasswren treatment was given, a female was observed in 35 of those trials (Table S 5.3). The sex of the responding individual was known in 38 trials; both a male and female were the first to respond in 4 trials (*A. m. indulkanna* = 3 and *A. m. raglessi* = 1), a male responded alone in 15 trials (*A. m. indulkanna* = 10 and *A. m. raglessi* = 5) and a female responded alone in 4 trials (*A. m. indulkanna* = 2 and *A. m. raglessi* =2). No territorial interactions with natural intruders were observed during playback experiments.

We performed 122 playback trials using three treatment types to *A. m. indulkanna* (n = 54) and *A. m. raglessi* (n = 68). Both subspecies had a low proportion of response to control song (< 5%) and were more likely to respond to either grasswren song treatments (own and other) than heterospecific song (Table 5.2, Figure 5.3). A*mytornis modestus raglessi* had a similar proportion of response towards either grasswren song treatment when accounting for random effects (Table 5.2, Figure 5.3). *Amytornis modestus indulkanna* responded ~15% more frequently to other grasswren song compared to own grasswren song (Table 5.2, Figure 5.3). When the fixed effect (treatment type) was removed from both focal subspecies models, there were no significant correlations between proportion of response and the random effects (*A. m. indulkanna*: intercept estimate = 0.06825, SE = 0.92783, z = -0.074, p = 0.941; *A. m. raglessi*: intercept estimate = -1.2224, SE = 0.7673, z = -1.593, p = 0.111) indicating that treatment type was an important variable in both of the models.

Response intensity within subspecies

We performed a PCA on the response variables recorded during playback trials with grasswren stimuli to compare observed response intensity between the treatments within subspecies (*A. m. indulkanna,* own: n = 10, other: n = 15; *A. m. raglessi,* own: n = 13, other: n = 9). One principle component (PC1) was extracted that explained 46.1% of the variation in

the response variables. Latency to 20 m, latency to 10 m and minimum distance were correlated with each other so that an increase in these variables indicated a reduction in response intensity. The frequency of speaker crosses and the frequency of song heard from the focal bird were also correlated with each other so that an increase in these variables indicated an increase in response intensity. There was no significant difference in the intensity of response between own and other grasswren treatments for either TBGW subspecies when PC1, latency to 20 m, minimum distance, speaker crosses or song frerquency was used as a measure of response intensity (Table 5.2, Figure 5.4). *Amytornis modestus indulkanna* had a stronger response to other compared to own stimulus types when latency to 10 m was used as a measure of response intensity while the response intensity of *A. m. raglessi* was equal for both own or other stimulus types (Table 5.2, Figure 5.4).

Discussion

This study found that the behavioural response to intruder song was different between two parapatric TBGW subspecies. The subspecies *A. m. indulkanna* was more likely to respond to hetero-subspecific than con-subspecific intruder song whereas the subspecies *A. m. raglessi* was equally likely to respond to hetero-subspecific and con-subspecific intruder song. This was matched by the intensity of the response, which was also greatest for *A. m. indulkanna* towards hetero-subspecific intruder song. Both subspecies were more likely to respond to either TBGW stimulus than the zebra finch control. Previous studies showed that there were areas of high and low gene flow between the subspecies and that gene flow occurred asymmetrically from *A. m. indulkanna* to *A. m. raglessi* (Chapter 2; Chaper 4). In this study, there was a pattern of asymmetric behavioural response that matched the pattern of asymmetric gene flow. These results suggest that there could be a behavioural mechanism influencing gene flow between *A. m. indulkanna* and *A. m. raglessi*.

Territorial behaviour has rarely been described in grasswrens and TBGW behaviour has only recently been studied in detail (Louter 2016). Response to playback is often used to understand territorial behaviour in passerines (Cain and Langmore 2016; Christensen *et al.* 2009; Hall and Peters 2008). The results from this study suggest that both TBGW subspecies recognised con-subspecific and hetero-subspecific TBGW song, as they consistently responded to grasswren intruder song but not the song of the control species. Approach and song rate are often used as indicators of aggression in birds (Billerman and Carling 2017; den Hartog *et al.* 2008; Poesel and Nelson 2012). We observed that TBGWs responded to the broadcast of grasswren song by approaching the speaker, singing and crossing the speaker. However, the only variable that was able to distinguish between differences in reponse intensity between different intruder types was 10 m latency. Grasswrens are particularly difficult to observe because they are well camouflaged and remain low to the ground (Rowley and Russell 1997). The variable "latency to 10 m" was likely to be more accurate compared with "latency to 20m".

The status of responding individual/s is likely to affect the behavioural responses. TBGW groups include breeder and helper birds that are likely to display different responses to intruders (Louter 2016). Identifying the sex of grasswrens is difficult because the only sexually dimorphic trait of grasswrens is concealed under the wing; females have rufous flanks and males do not. This is usually very difficult to see in the field. In this study, we were unable to consistently determine the respondents' sex or dominance status and instead analysed overall responses to playback. Further research should focus on understanding whether there are different roles for singing or responding individuals based on sex or dominance status within the group. Such studies would be beneficial for understanding whether sex and dominance affect territorial behaviour within grasswrens and would be interesting to eludicate the the role of territoriality in the evolution of the Maluridae.

Territorial response to intruder song may be used to predict subspecies competitiveness and measure discrimination between hetero-subspecifics and con-subspecifics (Tobias *et al.* 2012; Kleindorfer *et al.* 2013). Territorial behaviour towards hetero-(sub)specifics is common between different hybridising taxa (Curé *et al.* 2010; Ribot *et al.* 2013). Reduced response to hetero-(sub)specifics is usually indicative of a behavioural barrier to gene flow (Curry and Patten 2016; Dingle *et al.* 2010). In this study, the two TBGW subspecies had different response patterns to subspecies-specific song. *Amytornis modestus indulkanna* was more likely to respond and responded more strongly to hetero-subspecific song compared to con-subspecific song, while *A. m. raglessi* was equally likely to respond and with similar intensity to hetero-subspecific song compared to con-subspecific song. We previously showed that there is asymmetric gene flow from *A. m. indulkanna* to *A. m. raglessi* (Chapter 2; Chapter 4). A greater response (proportion and intensity) to hetero-subspecific song compared to con-subspecific song by *A. m. indulkanna* indicates they are more discriminatory against potential mates and/or that they may be able to outcompete *A. m. raglessi* in the core area of their distribution. Such local patterns of discrimination have been shown in other studies of avian parapatric species and subspecies where differences in competive behaviour were associated with gene flow asymmetries (Dingle *et al.* 2010; Pearson and Rohwer 2000); however, an increase in competitive behaviour is not always linked to an increase in gene flow (Billerman and Carling 2017; Freeman *et al.* 2016). Greater territorial response to intruders is more likely when 1) there is a large group size, 2) individuals are breeding, or 3) a female is present (Beletsky *et al.* 1990). Where possible, we recorded group size, breeding stage and sex of individuals present during playback experiments. It is likely that these factors were comparable between the two grasswren subspecies due to the random selection of territories used in this study and because playback experiments for each subspecies were performed within the same time of year. Much work

remains to be done to understand why the different subspecies would show different local patterns of response to an intruder bird from different subspecies.

Preliminary analysis showed that song from the two TBGW subspecies, *A. m. raglessi* and *A. m. indulkanna*, had different elements. Songs recorded from *A. m. raglessi* had 6/24 (25%) unique elements (i.e, one quarter of its vocal repertoire was unique to that subspecies) and songs recorded from *A. m. indulkanna* had 2/24 (8%) unique elements. Therefore, in theory, hetero-subspecific and con-subspecific song of intruder birds could be discriminated by territory holders based on differences in song composition. We previously showed that there is asymmetric introgression from *A. m. indulkanna* into *A. m. raglessi* (Chapter 2; Chapter 4). If this asymmetry is due to better dispersal propensity for *A. m. indulkanna*, *A. m. raglessi* offspring may be more exposed to the song of *A. m. indulkanna* than vice versa. Presuming that grasswrens are similar to fairy-wrens in that they are vocal learners (Evans and Kleindorfer 2016; Kleindorfer *et al.* 2014a), *A. m. raglessi* offspring may have learned more elements from *A. m. indulkanna* than vice versa causing *A. m. indulkanna* to have less unique elements than *A. m. raglessi*. Asymmetric introgression and asymmetric learning of hetero-subspecific song is also correlated in another species (Halfwerk *et al.* 2016).

Perhaps *A. m. indulkanna* responded more strongly to song from *A. m. raglessi* because songs from *A. m. raglessi* contained more unique elements. Novel signals have been shown to elicit stronger responses among different animal populations (Hamao 2016; Ord and Stamps 2009). High gene flow from *A. m. indulkanna* and *A. m. raglessi* was previously found within a region of parapatry (Chapter 4). We were unable to record song from individuals in the region of parapatry due to poor weather conditions and low levels of grasswren activity. If novel song elements are driving the observed difference in response probability and gene

flow, then we predict that hybridisation in the region of parapatry could be due to similar response patterns to song from either subspecies.

This study recorded song from two subspecies of TBGW and used those songs to measure subspecies response to hetero-subspecific and con-subspecific playback. *A. m. raglessi* song contained more unique vocal elements compared with *A. m. indulkanna* song (20% vs 8%). Both subspecies responded strongly to intruder song, but the response to hetero-subspecific playback compared to con-subspecific playback was more likely and stronger in *A. m. indulkanna* than in *A. m. raglessi*. The observed differences in territorial behaviour align with our previously published findings of asymmetrical gene flow: the subspecies with low genetic introgression was intolerant of hetero-subspecific intruders whereas the subspecies with higher genetic admixture were as likely to response to either intruder subspecies. These findings show different behavioural response patterns in diverged subspecies that are hybridising. At an early stage of speciation, these behavioural differences may be important for the development of future genetic barriers.

Acknowledgements

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providing example R scripts for analysing differences in proportion of response and response intensity. Finally, we are grateful to all the dedicated volunteers who helped with fieldwork.

Table 5.1 Variable loading and proportion of variance for PC1 (response intensity).

Eigenvalue	**2.31**
Percent variation	**46.1%**
Latency to 20 m	0.578
Latency to 10 m	0.611
Minimum distance	0.482
Number of crosses	-0.152
Number of songs	-0.192

Table 5.2 Results of generalised linear mixed models showing territorial response frequency and the response intensity of each focal thick-billed grasswren subspecies to different playback treatments. $P < 0.05$ are shown in bold.

			A. m. indulkanna				*A. m. raglessi*			
	Variable	Fixed effects	Estimate	SE	*z/t*	*p*	Estimate	SE	*z/t*	*p*
Proportion response	Presence/Absence	Intercept	25.529	6.823	3.741	**<0.001**	0.514	0.461	1.115	0.265
		Own vs Other	-14.050	5.139	-2.734	**0.006**	-0.853	0.700	-1.218	0.223
		Own vs Hetero	-39.177	10.148	-3.861	**<0.001**	-3.697	1.203	-3.073	**0.002**
		Other vs Hetero	-25.128	7.415	-3.389	**<0.001**	-2.844	1.155	-2.462	**0.014**
Response Intensity	PC1	Intercept	0.528	0.545	0.970	0.332	0.329	0.545	0.603	0.547
		Own vs Other	-1.141	0.957	-1.193	0.233	-0.134	0.830	-0.161	0.872
	10 m latency	Intercept	128.848	15.511	8.307	**<0.001**	97.035	21.063	4.607	**<0.001**
		Own vs Other	-53.338	18.198	-2.931	**0.003**	10.873	31.918	0.341	0.733
	20 m latency	Intercept	104.408	15.941	6.550	**<0.001**	89.663	18.989	4.722	**<0.001**
		Own vs Other	-34.090	27.153	-1.255	0.209	-0.314	28.964	-0.011	0.991
	min distance	Intercept	5.743	3.966	1.448	0.148	7.409	2.773	2.672	**0.007**
		Own vs Other	-3.743	7.920	-0.473	0.636	-3.072	1.807	-1.701	0.089
	speaker crosses	Intercept	2.408	0.572	4.206	**<0.001**	1.857	0.595	3.123	**0.002**
		Own vs Other	0.173	0.628	0.276	0.782	-0.413	0.951	-0.434	0.664

song frequency	Intercept	4.916	1.778	2.766	**0.006**	0.689	1.145	0.602	0.547
	Own vs Other	-2.940	3.808	-0.772	0.440	0.987	1.596	0.618	0.536

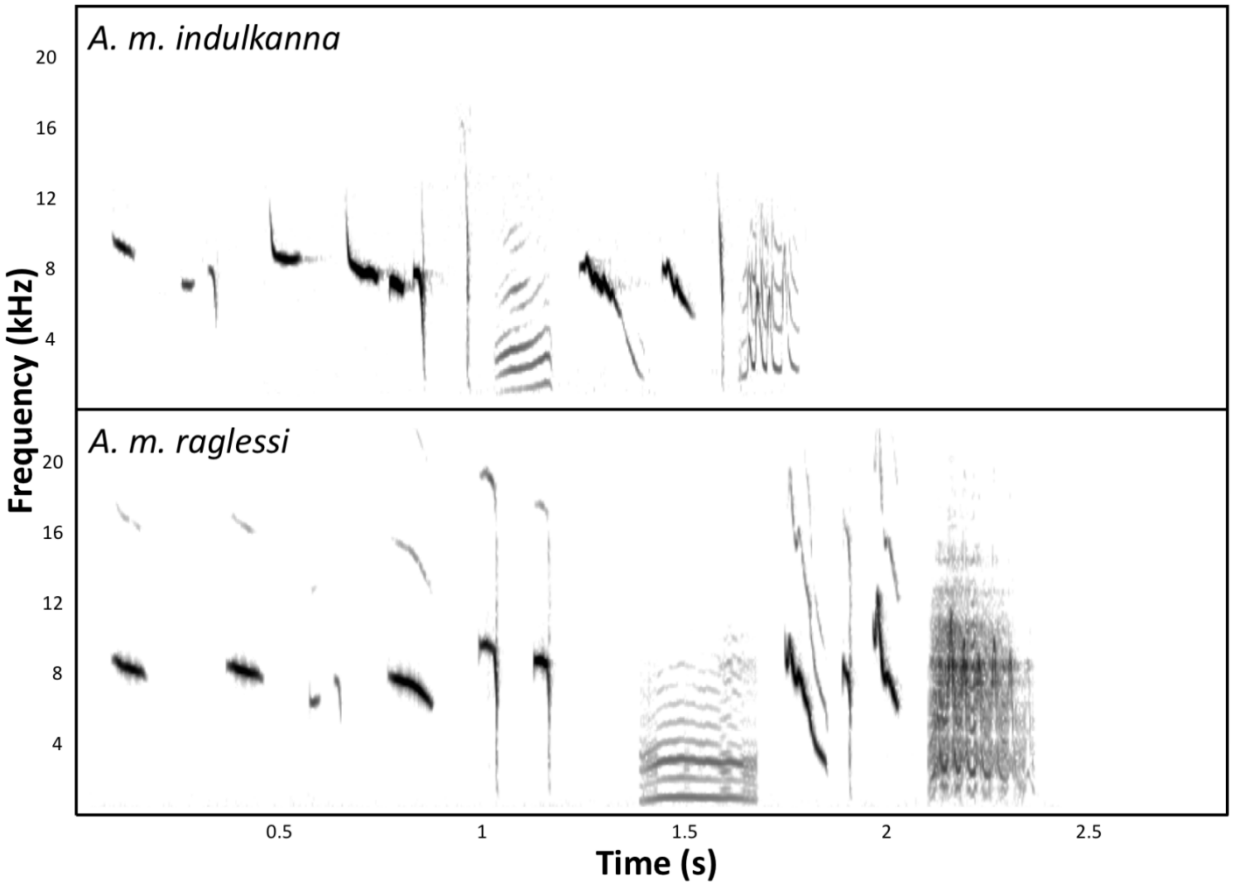

Figure 5.1 Examples of spectrograms of song from two thick-billed grasswren (*Amytornis modestus*) subspecies that were used for playback experiments.

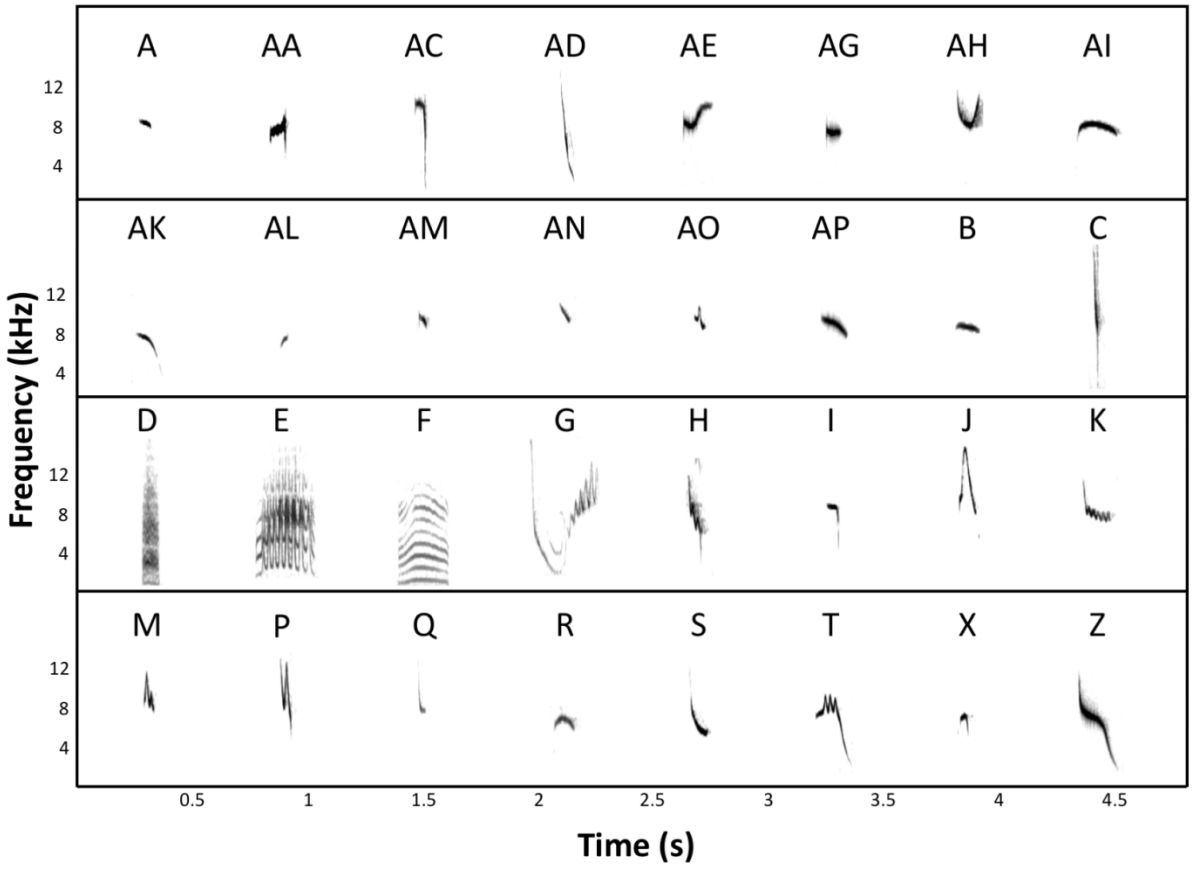

Figure 5.2 Spectrograms of different element types identified in 38 songs from two thick-billed grasswren (*Amytornis modestus*) subspecies; 24 element types were shared between song of the two subspecies, 6 element types were unique to *A. m. raglessi* song (AD, AE, AI, AK, AO and J) and 2 element types were unique to *A. m. indulkanna* song (T and X).

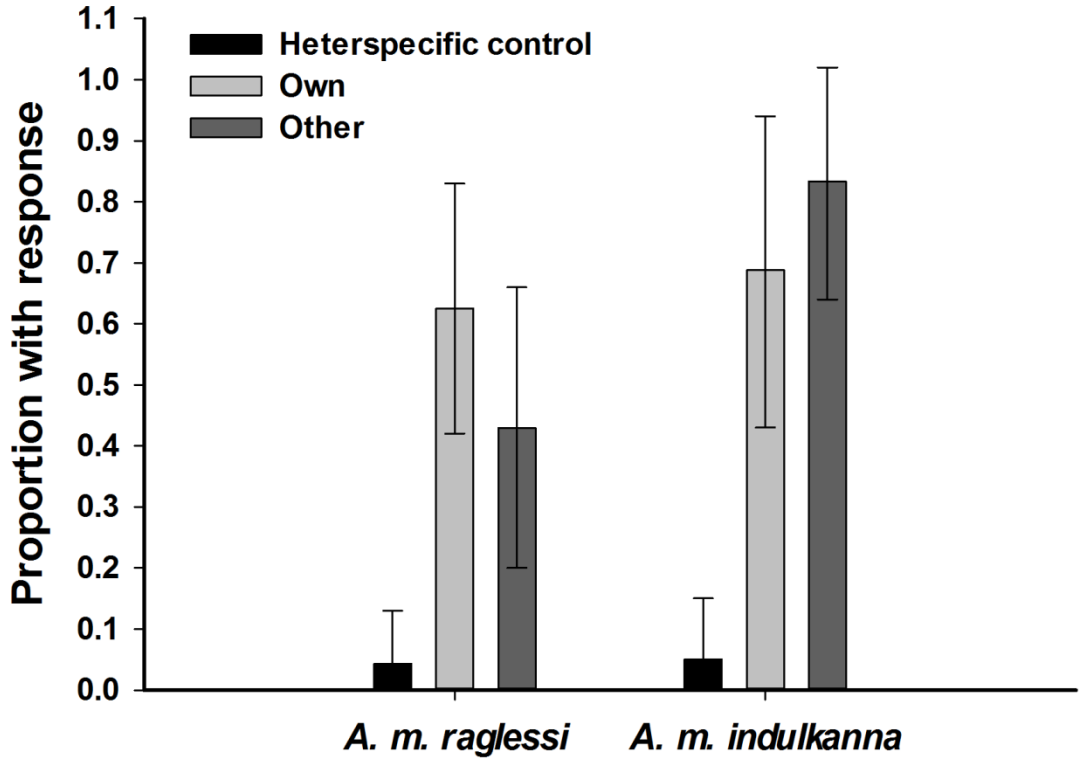

Figure 5.3 The proportion of territory-owners that responded to the experimental broadcast of intruder song in two thick-billed grasswren (*Amytornis modestus*) subspecies. "Own" refers to con-subspecific playback stimuli and "other" refers to hetero-subspecific playback stimuli. Error bars are binomial 95% confidence intervals. Sample sizes for *A. m. raglessi* are Own = 24, Other = 21 and Heterospecific control = 23. Sample sizes for *A. m. indulkanna* are Own = 16, Other = 18 and Heterospecific control = 20.

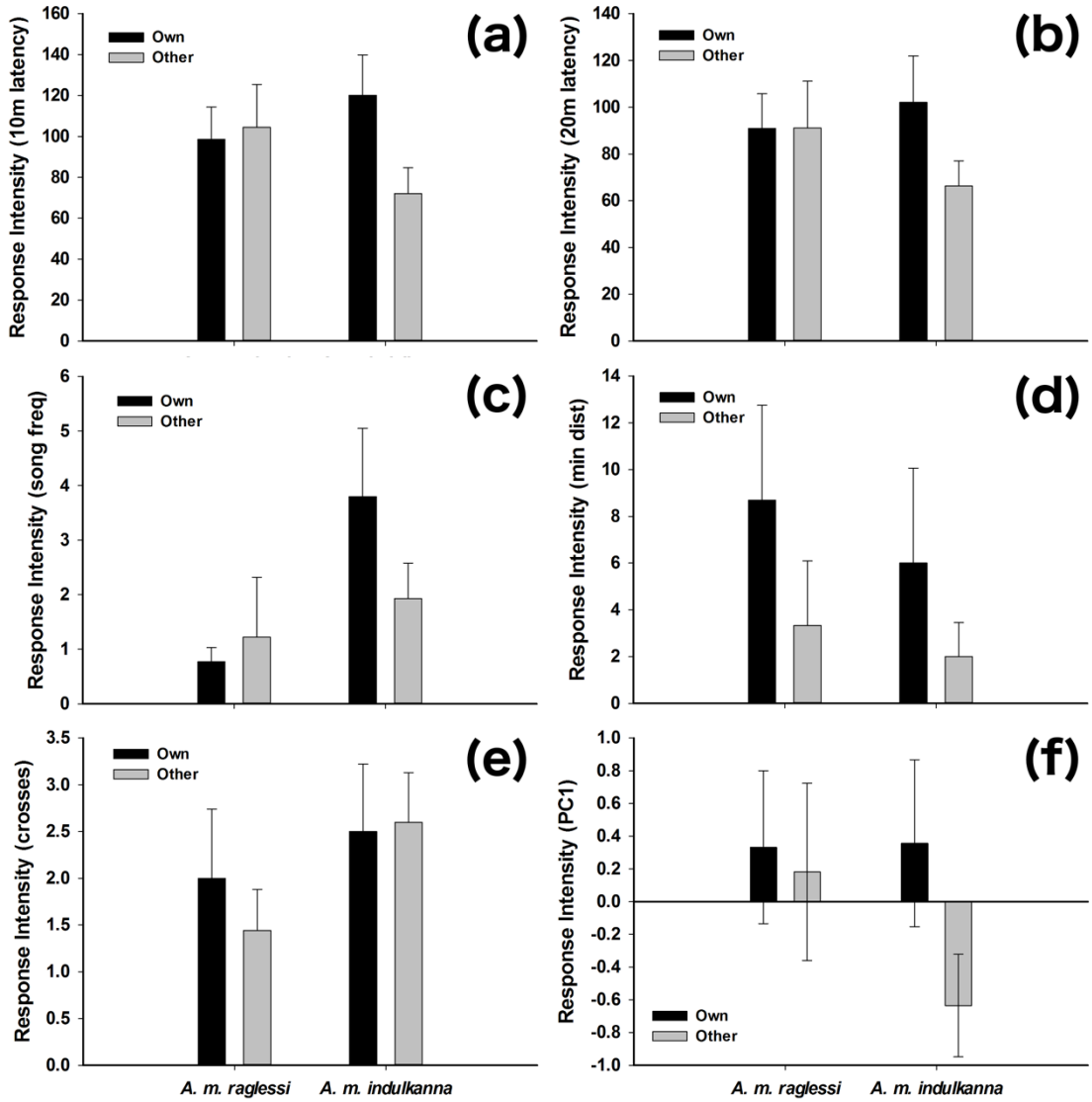

Figure 5.4 The mean territorial response intensity during playback trials to intruder song using univariate and multivariate response variables: (a) latency to approach within 10 m of the playback, (b) latency to approach within 20 m of the playback, (c) frequency of song heard from the target individual, (d) minimum distance to the playback, (e) number of crosses over the playback and (f) PC1 incorporating all previous variables (where a negative score indicates a stronger territorial response). Error bars indicate standard error of the mean. The response strength is shown for two thick-billed grasswren (*Amytornis modestus*) subspecies to different playback stimuli in playback trials where there was a positive binomial response. "Own" refers to con-subspecific playback stimuli and "other" refers to hetero-subspecific

playback stimuli. Sample sizes for *A. m. raglessi* are: Own = 13 and Other = 9. Sample sizes for *A. m. indulkanna* are Own = 10 and Other = 15.

Table S 5.1 Number of thick-billed grasswren groups and individuals used for song recordings and playback experiments at each location.

Location	Stimulus type	Latitude	Longitude	Groups used in playback*	Individuals/groups that produced song**	Stimuli (*n*)	Experiments with song from location (*n*)
Witchelina	zebra finch	30°01'S	138°03'E	N/A	1	1	16
Millbrook	zebra finch	N/A	N/A	N/A	11	11	27
Mt Lyndhurst	*A. m. raglessi*	30°11'S	138°43'E	2	0	N/A	N/A
Witchelina	*A. m. raglessi*	30°01'S	138°03'E	38	12	20	42
Coward Springs	*A. m. indulkanna*	29°24'S	136°49'E	9	3	6	14
Coober Pedy	*A. m. indulkanna*	29°01'S	134°45'E	8	3	3	7
Oodnadatta	*A. m. indulkanna*	27°34'S	135°27'E	5	1	2	4
Peculiar Knob	*A. m. indulkanna*	29°39'S	135°22'E	0	2	4	8
William Creek	*A. m. indulkanna*	30°01'S	138°03'E	7	2	3	4

* Different playback stimulus (hetero-subspecific, con-subspecific, control) performed for unbanded individuals at the same location across different years were classified as different groups

**Song recorded from unbanded individuals at the same location across different years were classified as originating from different individuals

Table S 5.2 Description of recordings from each thick-billed grasswren subspecies including the number of times a pair was observed, the total number of stimuli used, the number of stimuli where a pair was observed, the breeding stage of each stimuli (NB = nest building, INC = incubating, FC = feeding chicks, FL = fledglings, UNK = unknown), and mean group size per stimulus (± SD).

Stimulus group	Recording	Pair observed	Total number of stimuli	Stimuli (*n*)						Mean group size/stimulus
				With pair	NB	INC	FC	FL	UNK	
A. m. raglessi	12	11	20	19	0	5	6	1	8	3.5 ± 1.5
A. m. indulkanna	11	8 (3 UNK)	18	12	0	0	1	6	11	2.5 ± 0.9

Table S 5.3 Description of focal thick-billed grasswren subspecies that were targeted during playback trails including the number of playback trials (*n*), the proportion of playback trials where a female was known to be present (FP), the breeding stage of the targeted group during the playback trial (NB = nest building, INC = incubating, FC = feeding chicks, FL = fledglings, UNK = unknown), and the group size of the targeted group during the playback trial.

Playback design		Playback details		Breeding stage (*n*)					Group size (*n*)						
Focal subspecies	Treatment	*n*	FP (%)	NB	INC	FC	FL	UNK	One	Two	Three	Four	Five	Six	Eight
A. m. raglessi	own	24	42	2	3	3	7	10	2	6	3	8	3	2	1
A. m. raglessi	other	21	38	0	2	2	3	13	2	9	3	4	2	2	1
A. m. indulkanna	own	16	44	0	1	1	1	13	2	8	3	2	1	0	0
A. m. indulkanna	other	18	56	0	0	1	2	15	2	8	6	2	0	0	0

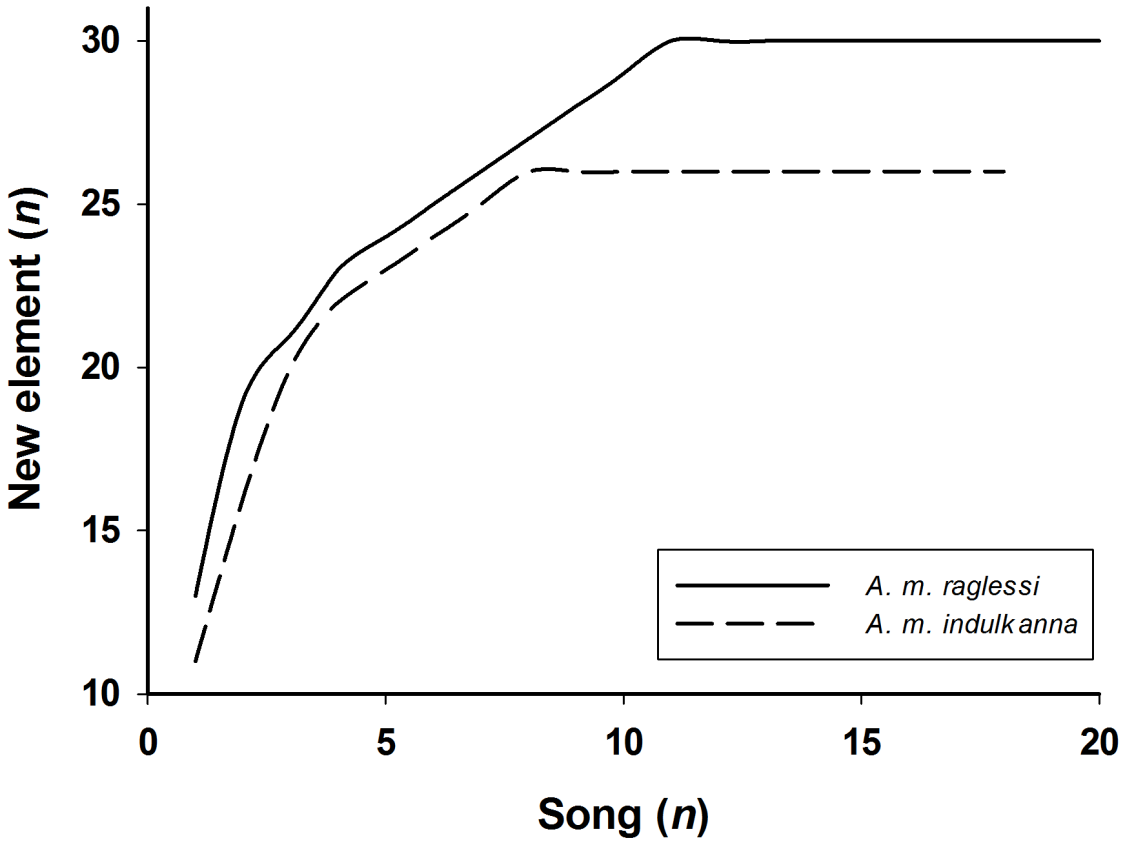

Figure S 5.1 Asymptotic curve showing the number of new elements discovered for each new song recording for *A. m. indulkanna* (dash) and *A. m. raglessi* (unbroken).

Chapter 6 General Discussion

Questions such as where do species come from and why do they exist in their current locations are pertinent for understanding evolution and classifying biodiversity. This has implications for conservation and land management. The songbirds (oscine) are a unique system for studying evolution because they are the most diverse avian clade and their radiation has only been relatively recent (Barker *et al.* 2004). Evolutionary studies of the songbirds are skewed towards systems that occur in the northern hemisphere even though it has been shown that the most recent common ancestor of the songbirds originated in Australasia (Ericson *et al.* 2002). The Maluridae are a particularly good group for studying both evolution within the Australian songbird fauna and conservation management as they have a variety of behaviours, ecologies and face a range of extinction pressures (Buchanan and Cockburn 2013; Skroblin and Murphy 2013). Fairywren species occurring in mesic environments are widely studied and understanding evolutionary processes that have affected the Maluridae would benefit by studying a greater breadth of systems (Joseph and Omland 2009). My thesis adds to our current understanding of evolutionary dynamics by studying the molecular ecology of an Australian songbird of the Maluridae family that occurs in an arid environment, the thick-billed grasswren (*Amytornis modestus*). The findings of this thesis describe four important features of two thick-billed grasswren subspecies including their distribution patterns (Chapter 2), ecotype distributions (Chapter 3), patterns of inter-subspecific gene flow (Chapter 4), and behavioural differences (Chapter 5). These findings provide an important example of evolutionary processes that increase our understanding of biodiversity.

Evolutionary Processes and Biodiversity

Subspecies with parapatric distributions are useful for discerning evolutionary processes that contribute to biodiversity and have implications for conservation management. I used the association between geographic location, morphotype and mitochondrial (mt) ND2 haplotype to identify the distribution pattern of two thick-billed grasswren (TBGW) subspecies (Chapter 2). If a single geographic region contained morphotypes and/or mt haplotypes that represent both subspecies, then it is possible that the subspecies have overlapping distributions. The presence of an overlapping region provides a pathway for inter-subspecific gene flow when there are no reproductive barriers between the subspecies. This is likely to affect factors that could either inhibit (introgression) or facilitate (greater genetic diversity) further population divergence. My research identified a potential region of parapatry between the two subspecies as well as evidence for inter-subspecific gene flow. While distinct morphotypes, which represent the two subspecies, did not occur within the same geographic region, the suspected region of parapatry contained male morphotypes that were intermediate between the subspecies as well as mt haplotypes from both eastern and western haplogroups. There was also discordance between morphotype and mt haplogroup within the region of parapatry and the core area for *A. m. raglessi* (characterised by distinct *A. m. raglessi* morphotype).

There were some limitations to the study. Grasswrens are particularly difficult to sample because they occur in regions with limited infrastructure and are difficult to find because they are shy and well camouflaged. The sample size within the predicted region of parapatry was small when separated by sex, and the distribution of the samples was not continuous across the landscape. Future research should focus on collecting more samples of each sex to increase the geographic coverage of sex-specific samples particularly in the region of

parapatry. This will allow further characterisation of the parapatric boundaries, identification of fine-scale landscape attributes that may contribute to patterns of inter-subspecific gene flow, and reveal associations between sex-specific dispersal and patterns of inter-subspecific gene flow. Sex-specific processes or fine-scale landscape processes may be important driving forces that affect species evolutionary trajectories.

Landscapes and parapatry

Ecological landscapes influence not only species ranges but also dispersal and survival that subsequently impact patterns of gene flow. I used associations between habitat type, geographic landscape and occurrence of TBGWs to determine how habitat type may affect boundaries between the region of parapatry and the core areas of two subspecies (Chapter 3). If the habitat type within the region of parapatry is distinct from the core areas of the subspecies or if the occurrence of TBGWs in the region of parapatry is dependent on habitat type, then there are two possible outcomes. Ecological heterogeneity within the region of parapatry could be associated with areas of high inter-subspecific gene flow and consequently high genetic diversity or ecological heterogeneity within the region of parapatry could be associated with areas of low inter-subspecific gene flow and subsequent divergence between the subspecies. This is the first time that a region of parapatry has been described for a grasswren species. My research showed that two distinct chenopod ecotypes occurred within the region of parapatry. Each chenopod ecotype was associated with the core area for either one of the subspecies. My research also showed that grasswren absence within the region of parapatry was associated with a distinct sand dune ecotype that extends along the entire boundary that separates the region of parapatry from the core area of *A. m. indulkanna*. There are limitations in any study that uses occurrence data to predict habitat–species associations because of the difficulty to consistently confirm absence throughout the day and

year. Further research should test TBGW preference for chenopod ecotypes over sand dune ecotypes as well as subspecies preference for different chenopod ecotypes. This would refine the prediction that ecological complexity across the landscape influences inter-subspecific gene flow.

Importance of hybridisation

The distribution of genetic variability across the landscape is useful for detecting processes related to both evolution and species persistence. This is the first study to examine nuclear genetic data in a grasswren species at the population level. I set out to quantify patterns of inter-subspecific gene flow across the region of parapatry and the core areas of the two subspecies (Chapter 4). If inter-subspecific gene flow in the core areas of the subspecies is low, then this could indicate a recent event leading to secondary contact or that the subspecies may be developing reproductive isolation, a marker of speciation. Otherwise, regions of high inter-subspecific gene flow could be related to increased species persistence due to the development of a broader range of phenotypes. My research showed that high inter-subspecific gene flow was limited to a narrow area within the suspected region of parapatry (Chapter 2). Inter-subspecific gene flow was asymmetric from *A. m. indulkanna* to *A. m. raglessi.* The ecotype within the core areas of the subspecies were different to each other and within the region of parapatry there was a mix of ecotypes associated with the grasswren occurance and subspecies type (Chapter 3). This suggests that ecological divergence along with heterogeneous ecotype distributions may contribute to subspecies divergence and genetic admixture.

Recent changes to dominant plant communities through anthropogenic use of the arid lands for grazing may have led to recent secondary contact, altering the evolutionary processes associated with these TBGW subspecies. Allopatric divergence may have led the subspecies

on a path towards further speciation but high genetic admixture can be related to greater local adaptation followed by divergence with gene flow. The main limitation of this study was the low sampling density across a large geographic area. This limitation means that detecting fine-scale spatial patterns of inter-subspecific gene flow such as those associated with short distance or sex-specific dispersal were not possible. To understand how historical events such as previous changes in climate have effected subspecies divergence and persistence, further research should test genetic models of migration, admixture and population size over time. Incorporated with models of future climate change, this will be useful for predicting how TBGW subspecies will respond to climate change in the future.

Behaviour and hybrid zones

Competition for resources can select for behaviours that affect gene flow. Divergent behaviours can impact processes related to evolution and threatened species management. I used experimental playback of TBGW song to test if territory-owners had a differentiated response to the song of intruders that were hetero- or con-subspecific birds (Chapter 5). If resident birds have a different response to intruder birds based on subspecies relationship, then behavioural mechanisms could shape different evolutionary patterns of mating and gene flow across subspecies and thereby increase the rate of population divergence. My research showed that *A. m. indulkanna* was more likely to response to hetero-subspecific compared to con-subspecific intruders while *A. m. raglessi* was equally likely to response to hetero- and con-subspecific intruders. These findings are parsimonious with patterns of asymmetrical gene flow documented in Chapter 4, whereby genetic introgression was greater in *A. m. raglessi* than *A. m. indulkanna*. This association may indicate a behavioural mechanism affecting contemporary patterns of gene flow. There are several limitations to the playback study. For example, this is the first study of TBGW song and it is possible that we did not

capture the complete vocal repertoire of each subspecies nor do we have a comprehensive understanding of song function in this species. The approach we used is standard in playback studies that broadcast song of one population to a resident bird and measure its response (Kroodsma 2005). This is the first time that differences in song between subspecies have been presented in this or any grasswren species. The song differed between subspecies as evidenced by the presence of unique elements in their song. Future research should aim to create a comprehensive song library for each subspecies, to experimentally test the function of song including possible roles in territory defence and mate attraction as well as test response of residents in the region of parapatry to *A. m. indulkanna* and *A. m. raglessi* song. This will highlight the role of song and behavioural response to song in predicting levels of inter-subspecific gene flow between parapatric subspecies.

Summary

The main priority of this thesis was to identify factors that affect evolutionary processes across a region of parapatry in an arid zone species. I found that ecological heterogeneity was the most important factor to influence inter-subspecific gene flow resulting in geographic regions with higher genetic diversity or higher genetic divergence. I also found behavioural patterns that suggest behavioural response selectivity may play a role in the direction of introgression. Barriers to gene flow are important for predicting subspecies trajectories for evolving and persisting especially when the subspecies have parapatric distributions. This process may require divergence of particular traits that have the power to overcome the homogenising effects of gene flow whilst contributing to the development of reproductive isolation (Smadja and Butlin 2011). The findings in this thesis suggests that landscape barriers (sand dunes) may contribute to selection for these particular traits. There are a variety of factors that are likely to affect the development of reproductive isolation. There is a need

for further research that clarifies processes that lead to reproductive isolation, such as whether reproductive isolation is caused by prezygotic or postzygotic barriers (Network 2012). Parapatric boundaries are useful for answering this question because differences between a region of parapatry and the regions outside the parapatric boundaries are likely to indicate processes affecting gene flow (Seehausen *et al.* 2014). I found that heterogeneous landscapes can reduce inter-subspecific gene flow when habitat patches are unsuitable for either one or both divergent subspecies. This could indicate that differential adaptation could be the first step leading to speciation between subspecies. Therefore, postzygotic barriers, through low hybrid fitness in suboptimal habitat, may be an important factor in the development of reproductive isolation. Ecological heterogeneity and differential adaptation have been identified as an important source of reproductive isolation in other environments (Beheregaray *et al.* 2015; Butlin *et al.* 2014; Edelaar *et al.* 2012). Ecological heterogeneity may be a particularly important factor in the first step of speciation with gene flow (Feder *et al.* 2012). The implications of the finding from this thesis suggest that an ecologically heterogeneous landscape is an important factor that affects evolutionary processes.

The second priority of this thesis was to determine patterns of inter-subspecific gene flow in order to develop appropriate conservation management strategies. Identifying patterns of inter-subspecific gene flow is useful for conservation management because 1) it reveals the appropriate taxonomic level of the group for conservation management, 2) it uncovers locations where individuals have greater genetic diversity, which can be more valuable for conservation management, and 3) it shows landscape features within populations that may create fragmentation and affect species persistence. I found that within the region of parapatry, the two TBGW subspecies were highly admixed and within the core area for *A. m. raglessi* there was evidence for hybrids that were greater than an F2 generation; there was discordance between mitochondrial haplotype and nuclear genetic background. In northern

hemisphere birds particularly, classification of subspecies based on mitochondrial differentiation has been misleading for conservation management (Phillimore and Owens 2006; Zink 2004). We recognised the two TBGW subspecies as distinct genetic lineages using nuclear genomic markers. In the absence of a complete reproductive barrier, the classification of subspecies is likely to be appropriate for these divergent lineages. Geographic regions of genetic admixture provide a source for increasing genetic variation that could lead to adaptation to changing environments and greater persistence (Hedrick 2013; Huang 2016; Pavlova *et al.* 2014). The region of parapatry and the core area for *A. m. raglessi* showed high to low levels of inter-subspecific gene flow respectively. This could mean that individuals in these areas are valuable for future conservation management. Landscape features that prevent gene flow can reduce genetic variability in isolated areas (Harrisson *et al.* 2013). I found that sand dunes are one such landscape feature that may create areas with reduced gene flow. Although the dune field that we identified as a landscape barrier is currently permeable, a drier and windier climate may increase the strength of this barrier and create other barriers where sand dunes accumulate. Accounting for these risks and developing appropriate management strategies are necessary when conserving threatened subspecies especially when climate is predicted to become much warmer in the future.

Conclusion

Using data that incorporates multiple evolutionary fields such as ecology, molecular biology, and behaviour, I have described features that may have affected divergence and introgression in two TBGW subspecies. This is an important system for studying evolutionary processes because TBGWs are an Australian songbird of the Maluridae that occur in arid environments and are threatened with extinction. The Australian arid zone has a history of considerable

climate change from periods that were wetter and more humid to periods that were drier and windier. Predictions about future climate change suggest that severe weather, such as droughts and heat waves, will become more frequent and will greatly affect the distribution and persistence of many species (Morán-Ordóñez *et al.* 2018). Changing climate may have affected evolutionary processes in the TBGW and may continue to do so in the future. TBGWs have other interesting characteristics worthy of further research such as an association between territorial aggression and asymmetric introgression. Whether and how these characteristics affect the development of reproductive isolation between these subspecies and their persistence in the future will be important for conservation management.

TBGWs appear to be in the early stages of divergence where differences in morphology are small but significant and genetic differentiation of nuclear markers is low. The narrow region of high genetic admixture suggests that the two TBGW subspecies could continue to diverge with gene flow but that this may depend on the heterogeneous distribution of different ecotypes. TBGWs are vulnerable to extinction because they have had a reduction in distribution due to overgrazing. Areas of high inter-subspecific gene flow are likely to contain a considerable amount of genetic variation that may be crucial for the continued persistence of TBGWs in a landscape that is likely to be impacted in the future by climate change.

Appendix

Details of samples used in the study. The first 118 samples are listed in the same order as in Figure 4.3. Location codes are the same as in Figure 4.1. Abbreviations in sample codes: ABBBS – Australian Bird and Bat Banding Scheme, ANWC – Australian National Wildlife Collection, Canberra, SAMA – South Australian Museum, Adelaide., m – male, f – female. Other abbreviations: ND – not done, N/A – not applicable.

Sample	Year	Site	Zone	Location	Location code	Sex	PC1: body size	PC2: bill shape	PC3: body shape	mtDNA Clade	mtDNA Haplotype	Genbank Accession Number	Used in Chapter 4	Used in Chapter 2
SAMA BS7001	2008	32	A	Oodnadatta West	OOW	ND	ND	ND	ND	West	26	KY059941	Y	Y
ABBBS 03666538	2013	30	A	Oodnadatta West	OOW	f	0.50	-0.87	-1.86	West	5	KY059987	Y	Y
ABBBS 03666537	2013	30	A	Oodnadatta West	OOW	m	1.56	-0.90	-0.07	West	24	KY059949	Y	Y
ABBBS 03666541	2013	28	A	Oodnadatta West	OOW	f	0.35	-0.42	-1.20	West	8	KY059972	Y	Y
ABBBS 03666540	2013	29	A	Oodnadatta West	OOW	f	ND	ND	ND	West	23	KY059952	Y	Y
ABBBS 03666539	2013	29	A	Oodnadatta West	OOW	m	0.84	0.23	0.25	West	8	KY059973	Y	Y
ABBBS 03666534	2013	31	A	Oodnadatta West	OOW	m	1.66	-0.94	0.88	West	5	KY059989	Y	Y
SAMA B55706	2007	34	A	Oodnadatta East	OOE	f	ND	ND	ND	West	17	KF053468*	Y	Y

SAMA B55705	2007	33	A	Oodnadatta East	OOE	f	ND	ND	ND	West	13	KF053467*	Y	Y
SAMA B55707	2007	33	A	Mount Barry station	MTB	f	ND	ND	ND	West	5	KF053469*	Y	Y
SAMA B55669	2007	19	A	Coober Pedy	COP	m	ND	ND	ND	West	5	KF053465*	Y	Y
ABBBS 03666570	2013	16	A	Coober Pedy	COP	m	1.68	-0.47	1.09	West	8	KY059970	Y	Y
ABBBS 03666555	2013	18	A	Coober Pedy	COP	m	1.47	-0.75	0.47	West	5	KY059986	Y	Y
ABBBS 03666554	2013	18	A	Coober Pedy	COP	f	0.77	-1.39	-0.45	West	1	KY059991	Y	Y
ABBBS 03666574	2013	14	A	Coober Pedy	COP	m	0.82	-0.43	0.45	West	21	KY059947	Y	Y
ABBBS 03666573	2013	14	A	Coober Pedy	COP	f	1.14	-1.12	-0.60	West	8	KY059969	Y	Y
ABBBS 03666572	2013	15	A	Coober Pedy	COP	f	1.11	-1.40	-1.30	West	19	KY059943	Y	Y
ABBBS 03666571	2013	15	A	Coober Pedy	COP	m	-0.29	-1.14	-0.53	West	15	KY059961	Y	Y
ABBBS 03666562	2013	17	A	Coober Pedy	COP	m	0.66	-1.06	0.56	West	4	KY059984	Y	Y
ABBBS 03666557	2013	20	A	Peculiar Knob	COP	m	1.59	-0.45	0.11	West	5	KY059985	Y	Y
ABBBS 03666560	2013	26	A	Peculiar Knob	PEK	f	ND	ND	ND	West	16	KY059962	Y	Y
ABBBS 03666559	2013	24	A	Peculiar Knob	PEK	m	0.64	-0.79	-0.44	West	10	KY059983	Y	Y
ABBBS 03666558	2013	24	A	Peculiar Knob	PEK	f	0.04	-1.31	-1.60	West	9	KY059937	Y	Y
ABBBS 03666566	2013	23	A	Peculiar Knob	PEK	f	1.46	-0.50	-1.20	West	23	KY059948	Y	Y
ABBBS 03666561	2013	25	A	Peculiar Knob	PEK	m	1.11	-0.85	0.34	West	6	KY059960	Y	Y
ABBBS 03666568	2013	21	A	Peculiar Knob	PEK	f	1.03	-1.58	-0.66	West	2	KY059992	Y	Y
SAMA B55670	2007	27	A	Peculiar Knob	PEK	m	ND	ND	ND	West	5	KF053466*	Y	Y
ABBBS 03666546	2013	13	A	William Creek	WIC	m	ND	ND	ND	West	2	KY059993	Y	Y
ABBBS 03666543	2013	10	A	William Creek	WIC	m	1.04	-0.83	-0.32	West	7	KY059963	Y	Y
ABBBS 03666548	2013	11	A	William Creek	WIC	f	-0.07	-0.95	-0.32	West	20	KY059946	Y	Y
ABBBS 03666552	2013	9	A	William Creek	WIC	f	0.44	-0.79	-1.73	West	23	KY059951	Y	Y
ABBBS 03666551	2013	9	A	William Creek	WIC	m	0.82	-0.36	0.32	West	8	KY059957	Y	Y
ABBBS 03666547	2013	12	A	William Creek	WIC	m	0.75	-0.76	-0.75	West	8	KY059971	Y	Y

SAMA B59006**	2013	5	A	Coward Springs Railway Siding	COS	f	ND	ND	ND	West	3	KY059994	Y	Y
ABBBS 03666582	2013	5	A	Coward Springs Railway Siding	COS	m	0.26	-1.47	-0.47	West	8	KY059966	Y	Y
ABBBS 03666577	2013	4	A	Coward Springs Railway Siding	COS	m	0.77	-0.26	-0.90	West	8	KY059968	Y	Y
ABBBS 03666585	2013	3	A	Coward Springs Railway Siding	COS	m	0.06	-1.00	0.60	West	8	KY059965	Y	Y
ABBBS 03666584	2013	3	A	Coward Springs Railway Siding	COS	f	-0.53	-1.54	-1.36	West	3	KY059995	Y	Y
ABBBS 03666580	2013	6	A	Coward Springs Railway Siding	COS	m	ND	ND	ND	West	17	KY059942	Y	Y
ABBBS 03666579	2013	6	A	Coward Springs Railway Siding	COS	f	-0.70	-1.89	-0.55	West	8	KY059967	Y	Y
ABBBS 03666576	2013	8	A	Coward Springs Railway Siding	COS	m	1.11	-1.10	-1.08	West	26	KY059940	Y	Y
ABBBS 03666575	2013	8	A	Coward Springs Railway Siding	COS	f	0.79	-2.00	-0.87	West	12	KY059955	Y	Y
SAMA B59004**	2013	7	A	Coward Springs Railway Siding	COS	m	2.24	-1.32	-0.80	West	25	KY059938	Y	Y
SAMA B59005**	2013	7	A	Coward Springs Railway Siding	COS	m	2.29	0.63	1.62	West	14	KY059956	Y	Y
SAMA B55668	2007	1	A	Andamooka	MUL	f	ND	ND	ND	West	8	KF053489*	Y	Y
SAMA B55667	2007	2	A	Andamooka	MUL	m	ND	ND	ND	West	13	KF053490*	Y	Y
SAMA B59003**	2013	44	AB	Mulgaria station	MUL	m	0.74	-0.21	0.17	West	23	KY059950	Y	Y
ABBBS 03666530	2014	35	AB	Stuart Creek	STC	m	-0.62	-1.44	1.75	West	23	KY059953	Y	Y

ABBBS 03666527	2014	36	AB	Stuart Creek	STC	f	1.52	-1.05	0.67	East	34	KY059933	Y	Y
ABBBS 03666529	2014	37	AB	Stuart Creek	STC	f	0.86	-0.83	-0.66	East	28	KY059865	Y	Y
ABBBS 03666528	2014	43	AB	Stuart Creek	STC	f	ND	ND	ND	West	5	KY059990	Y	Y
ABBBS 03666532	2014	42	AB	Stuart Creek	STC	f	ND	ND	ND	East	31	KY059907	Y	Y
ABBBS 03666518	2014	41	AB	Stuart Creek	STC	m	-0.80	-1.05	-0.78	East	33	KY059936	Y	Y
ABBBS 03666523	2014	38	AB	Stuart Creek	STC	m	0.26	-0.37	0.57	West	8	KY059958	Y	Y
ABBBS 03666522	2014	40	AB	Stuart Creek	STC	f	-0.24	-1.87	-1.65	East	30	KY059879	Y	Y
ABBBS 03666526	2014	39	AB	Stuart Creek	STC	f	0.19	-0.97	-0.45	East	30	KY059878	Y	Y
ABBBS 03666524	2014	39	AB	Stuart Creek	STC	m	-0.16	-0.82	-0.88	East	31	KY059908	Y	Y
ABBBS 03667351	2013	75	B	Witchelina Nature Reserve	WIT	f	-1.40	0.90	-0.66	East	36	KY059898	Y	Y
ABBBS 03667352	2013	75	B	Witchelina Nature Reserve	WIT	m	-0.67	1.14	-0.25	East	27	KY059875	Y	Y
ABBBS 03667396	2013	69	B	Witchelina Nature Reserve	WIT	m	-0.06	0.61	0.29	East	41	KY059905	Y	Y
ABBBS 03667397	2013	69	B	Witchelina Nature Reserve	WIT	f	-1.01	1.02	-0.02	West	25	KY059939	Y	Y
ABBBS 03666887	2013	66	B	Witchelina Nature Reserve	WIT	f	-1.42	0.73	-0.15	East	27	KY059873	Y	Y
ABBBS 03666886	2013	66	B	Witchelina Nature Reserve	WIT	m	0.91	0.49	1.13	West	8	KY059977	Y	Y
ABBBS 02572570	2012	65	B	Witchelina Nature Reserve	WIT	m	-0.99	1.23	-0.73	East	30	KY059881	Y	Y
ABBBS 03666589	2013	65	B	Witchelina Nature Reserve	WIT	m	0.11	0.36	0.84	West	8	KY059975	Y	Y
ABBBS 03666593	2014	77	B	Witchelina	WIT	m	ND	ND	ND	East	28	KY059870	Y	Y

				Nature Reserve									
ABBBS 03667362	2013	68	B	Witchelina Nature Reserve	WIT	m	1.23	1.57	-0.96	East	31	KY059921	Y	Y
ABBBS 03667393	2013	71	B	Witchelina Nature Reserve	WIT	m	-2.21	0.25	-0.06	West	8	KY059981	Y	Y
ABBBS 03667395	2013	71	B	Witchelina Nature Reserve	WIT	f	-1.23	1.15	-0.59	East	30	KY059885	Y	Y
ABBBS 03666899	2013	79	B	Witchelina Nature Reserve	WIT	m	0.94	1.70	1.30	East	41	KY059904	Y	Y
ABBBS 03667367	2013	56	B	Witchelina Nature Reserve	WIT	f	-0.59	1.17	0.61	East	30	KY059887	Y	Y
ABBBS 03667368	2013	57	B	Witchelina Nature Reserve	WIT	m	0.79	1.54	1.04	East	37	KY059893	Y	Y
ABBBS 03666888	2013	57	B	Witchelina Nature Reserve	WIT	f	-0.96	0.38	0.27	East	37	KY059891	Y	Y
NestT36	2013	89	B	Witchelina Nature Reserve	WIT	m	N/A	N/A	N/A	West	N/A	N/A	Y	N
ABBBS 03667387	2013	55	B	Witchelina Nature Reserve	WIT	f	-0.88	0.20	-0.09	East	31	KY059919	Y	Y
ABBBS 03666875	2013	58	B	Witchelina Nature Reserve	WIT	f	-1.67	-0.83	-0.60	West	8	KY059976	Y	Y
ABBBS 03666876	2013	58	B	Witchelina Nature Reserve	WIT	m	-0.30	0.37	1.40	East	31	KY059912	Y	Y
ABBBS 03636691	2014	51	B	Witchelina Nature Reserve	WIT	f	-0.19	-0.64	-1.33	West	7	KY059964	Y	Y
ABBBS 03636688	2014	50	B	Witchelina	WIT	m	-0.14	-0.62	0.27	East	31	KY059925	Y	Y

				Nature Reserve									
ABBBS 03636690	2014	50	B	Witchelina Nature Reserve	WIT	f	-0.76	-0.07	-1.13	East	35	KY059890	Y	Y
ABBBS 03636697	2014	53	B	Witchelina Nature Reserve	WIT	m	0.26	1.39	1.45	East	31	KY059928	Y	Y
ABBBS 03667374	2013	53	B	Witchelina Nature Reserve	WIT	f	-1.31	0.53	0.05	East	31	KY059923	Y	Y
ABBBS 03667371	2013	74	B	Witchelina Nature Reserve	WIT	m	0.31	1.37	1.65	East	31	KY059922	Y	Y
ABBBS 03667373	2013	74	B	Witchelina Nature Reserve	WIT	f	-0.12	1.21	-0.90	East	40	KY059902	Y	Y
ABBBS 03666590	2013	47	B	Witchelina Nature Reserve	WIT	m	1.78	0.83	1.76	East	40	KY059901	Y	Y
ABBBS 03667389	2013	48	B	Witchelina Nature Reserve	WIT	f	-1.42	-0.74	0.32	East	31	KY059920	Y	Y
ABBBS 03667383	2013	49	B	Witchelina Nature Reserve	WIT	f	-1.94	0.04	0.55	East	31	KY059918	Y	Y
ABBBS 03667384	2013	49	B	Witchelina Nature Reserve	WIT	m	-0.62	0.70	-0.66	West	8	KY059979	Y	Y
ABBBS 03636696	2014	47	B	Witchelina Nature Reserve	WIT	m	0.29	1.26	2.12	East	31	KY059927	Y	Y
ABBBS 02572568	2013	70	B	Witchelina Nature Reserve	WIT	m	ND	ND	ND	East	36	KY059894	Y	Y
ABBBS 03666891	2013	63	B	Witchelina Nature Reserve	WIT	m	1.14	0.85	1.24	East	28	KY059867	Y	Y
ABBBS 03666893	2013	63	B	Witchelina	WIT	f	-1.75	-0.43	-0.79	East	29	KY059876	Y	Y

				Nature Reserve									
ABBBS 02572572	2012	82	B	Witchelina Nature Reserve	WIT	m	ND	ND	ND	East	31	KY059910	Y	Y
ABBBS 03666897	2013	67	B	Witchelina Nature Reserve	WIT	f	-0.14	-0.30	-0.06	East	27	KY059874	Y	Y
ABBBS 03666898	2013	67	B	Witchelina Nature Reserve	WIT	m	0.60	0.36	0.66	East	31	KY059916	Y	Y
ABBBS 03667363	2013	78	B	Witchelina Nature Reserve	WIT	m	-0.73	0.93	-0.13	East	28	KY059869	Y	Y
ABBBS 03666588	2013	60	B	Witchelina Nature Reserve	WIT	m	0.41	-0.87	2.33	East	30	KY059882	Y	Y
ABBBS 03667347	2014	60	B	Witchelina Nature Reserve	WIT	f	-1.56	0.65	-1.84	East	40	KY059903	Y	Y
ABBBS 03666586	2013	54	B	Witchelina Nature Reserve	WIT	f	-0.72	-0.63	1.27	East	36	KY059896	Y	Y
SAMA B59025	2014	54	B	Witchelina Nature Reserve	WIT	m	-0.59	0.72	-1.59	East	36	KY059899	Y	Y
ABBBS 03667334	2014	62	B	Witchelina Nature Reserve	WIT	f	0.44	1.31	-0.24	East	31	KY059929	Y	Y
ABBBS 03667332	2014	62	B	Witchelina Nature Reserve	WIT	m	1.29	1.81	-0.12	East	31	KY059930	Y	Y
ABBBS 03667327	2014	76	B	Witchelina Nature Reserve	WIT	m	-0.72	0.67	0.01	East	31	KY059932	Y	Y
ABBBS 03667331	2014	90	B	Witchelina Nature Reserve	WIT	f	N/A	N/A	N/A	East	N/A	N/A	Y	N
ABBBS 03667381	2013	45	B	Witchelina	WIT	m	0.16	1.06	-1.67	West	8	KY059978	Y	Y

ABBBS 03636681	2014	46	B	Nature Reserve Witchelina Nature Reserve	WIT	m	N/A	N/A	N/A	East	N/A	N/A	Y	N
ABBBS 03666882	2013	59	B	Witchelina Nature Reserve	WIT	m	0.35	1.24	-0.51	East	31	KY059915	Y	Y
ABBBS 03667365	2013	73	B	Witchelina Nature Reserve	WIT	m	-0.85	1.22	-0.31	East	30	KY059886	Y	Y
ANWC B40189	1985	84	B	Witchelina Nature Reserve	WIT	m	ND	ND	ND	East	31	KF053482*	Y	Y
ANWC B40190	1985	84	B	Witchelina Nature Reserve	WIT	f	ND	ND	ND	East	37	KF053483*	Y	Y
ANWC B40177	1985	83	B	Mount Lyndhurst station	MTL	f	ND	ND	ND	East	28	KF053479*	Y	Y
ANWC B40180	1985	83	B	Mount Lyndhurst station	MTL	m	ND	ND	ND	East	32	KF053481*	Y	Y
ABBBS 03666512	2014	85	B	Mount Lyndhurst	MTL	m	-0.16	0.79	1.42	East	39	KY059864	Y	Y
ABBBS 03666511	2014	85	B	Mount Lyndhurst	MTL	f	-0.46	0.66	0.81	East	33	KY059934	Y	Y
ABBBS 03666514	2014	86	B	Mount Lyndhurst	MTL	m	0.17	0.53	0.72	East	39	KY059862	Y	Y
ABBBS 03666513	2014	86	B	Mount Lyndhurst	MTL	f	-0.54	-0.65	-1.19	East	39	KY059863	Y	Y
SAMA B55666	2007	87	B	Murnpeowie	MUR	m	ND	ND	ND	East	31	KF053485*	Y	Y

				station									
SAMA B56154	2009	88	B	Murnpeowie station	MUR	f	ND	ND	ND	East	31	KF053486*	Y	Y
ABBBS 03666535	2013	31	A	Oodnadatta West	OOW	m	0.71	-0.98	0.42	West	5	KY059988	N	Y
ABBBS 03666569	2013	16	A	Coober Pedy	COP	m	ND	ND	ND	West	18	KY059944	N	Y
ABBBS 03666563	2013	17	A	Coober Pedy	COP	f	ND	ND	ND	West	6	KY059959	N	Y
ABBBS 03666565	2013	22	A	Peculiar Knob	PEK	m	0.86	-0.88	0.50	West	10	KY059982	N	Y
ABBBS 03666564	2013	22	A	Peculiar Knob	PEK	f	ND	ND	ND	West	11	KY059954	N	Y
ABBBS 03666545	2013	10	A	William Creek	WIC	m	ND	ND	ND	West	22	KY059945	N	Y
ABBBS 03666583	2013	5	A	Coward Springs Railway Siding	COS	m	0.66	-1.21	-0.96	West	3	KY059996	N	Y
ABBBS 03666531	2014	35	AB	Stuart Creek	STC	f	ND	ND	ND	East	30	KY059877	N	Y
ABBBS 03666521	2014	41	AB	Stuart Creek	STC	m	-0.76	-0.99	-0.92	East	33	KY059935	N	Y
ABBBS 03666520	2014	41	AB	Stuart Creek	STC	f	ND	ND	ND	West	8	KY059974	N	Y
ABBBS 03667382	2013	45	B	Witchelina Nature Reserve	WIT	f	ND	ND	ND	East	31	KY059917	N	Y
ABBBS 02572577	2012	46	B	Witchelina Nature Reserve	WIT	m	-0.66	-0.13	0.64	East	28	KY059866	N	Y
ABBBS 03666599	2014	46	B	Witchelina Nature Reserve	WIT	f	-1.55	1.15	-0.02	ND	ND	ND	N	Y
ABBBS 03636683	2014	46	B	Witchelina Nature Reserve	WIT	f	-2.22	-0.52	-1.53	East	31	KY059924	N	Y
ABBBS 03667399	2013	47	B	Witchelina Nature Reserve	WIT	f	0.41	-0.04	0.29	East	31	KY059911	N	Y
ABBBS 03636695	2014	47	B	Witchelina	WIT	m	0.39	1.41	0.27	East	31	KY059926	N	Y

				Nature Reserve									
ABBBS 03667379	2013	48	B	Witchelina Nature Reserve	WIT	m	0.74	1.28	2.22	East	28	KY059868	N	Y
ABBBS 03636689	2014	50	B	Witchelina Nature Reserve	WIT	f	-1.85	0.32	-0.87	ND	ND	ND	N	Y
ABBBS 03667369	2013	51	B	Witchelina Nature Reserve	WIT	m	-0.01	1.39	0.45	East	35	KY059889	N	Y
ABBBS 02572575	2012	52	B	Witchelina Nature Reserve	WIT	m	-0.74	-0.05	0.14	East	27	KY059872	N	Y
ABBBS 02572574	2012	52	B	Witchelina Nature Reserve	WIT	f	-1.60	-0.80	0.64	East	35	KY059888	N	Y
ABBBS 03636686	2014	52	B	Witchelina Nature Reserve	WIT	m	-0.02	1.40	-0.82	East	38	KY059861	N	Y
ABBBS 03666587	2013	54	B	Witchelina Nature Reserve	WIT	m	-0.27	-0.67	2.83	East	36	KY059895	N	Y
ABBBS 03667366	2013	56	B	Witchelina Nature Reserve	WIT	m	-1.20	1.21	1.15	East	37	KY059892	N	Y
ABBBS 03666877	2013	58	B	Witchelina Nature Reserve	WIT	m	0.91	0.65	1.87	East	31	KY059913	N	Y
ABBBS 03666881	2013	59	B	Witchelina Nature Reserve	WIT	f	-0.83	0.41	-0.17	East	31	KY059914	N	Y
ABBBS 03667340	2014	61	B	Witchelina Nature Reserve	WIT	m	-0.03	1.40	0.98	East	36	KY059900	N	Y
ABBBS 02572571	2012	64	B	Witchelina Nature Reserve	WIT	m	-0.05	2.13	0.99	East	30	KY059880	N	Y
ABBBS 03667375	2013	72	B	Witchelina	WIT	f	-1.12	0.15	-0.87	East	30	KY059884	N	Y

				Nature Reserve									
ABBBS 03667376	2013	72	B	Witchelina Nature Reserve	WIT	m	-0.85	0.80	0.57	West	8	KY059980	N	Y
ABBBS 03667377	2013	73	B	Witchelina Nature Reserve	WIT	m	ND	ND	ND	East	41	KY059906	N	Y
ABBBS 03667329	2014	76	B	Witchelina Nature Reserve	WIT	f	-0.31	1.37	-0.58	East	31	KY059931	N	Y
ABBBS 03667364	2013	78	B	Witchelina Nature Reserve	WIT	f	-1.80	0.67	0.56	East	36	KY059897	N	Y
ABBBS 03636687	2014	78	B	Witchelina Nature Reserve	WIT	f	ND	ND	ND	East	28	KY059871	N	Y
ABBBS 03666900	2013	80	B	Witchelina Nature Reserve	WIT	m	ND	ND	ND	East	30	KY059883	N	Y
ABBBS 02572576	2012	81	B	Witchelina Nature Reserve	WIT	m	ND	ND	ND	East	31	KY059909	N	Y
ANWC B40192	1985	84	B	Witchelina Nature Reserve	WIT	f	ND	ND	ND	East	37	KF053484*	N	Y
ANWC B40176	1985	83	B	Mount Lyndhurst station	MTL	m	ND	ND	ND	East	30	KF053478*	N	Y
ANWC B40179	1985	83	B	Mount Lyndhurst station	MTL	f	ND	ND	ND	East	32	KF053480*	N	Y

*Sequences from (Austin *et al.* 2013)

**Samples subsequently submitted to the South Australian museum after sample collection for this study.

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