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The causes & evolutionary consequences of behavioural, morphological & molecular genetic variation in the Chowchilla, *Orthonyx spaldingii*.

Thesis submitted by
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in May 2007

For the degree of Doctor of Philosophy
in the School of Marine & Tropical Biology
James Cook University

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The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human (*1999*),* the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997*),* the *James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A870_04).

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(Signature)   (Date)

Acknowledgements

" A first encounter with Chowchillas may occur without a single bird in view, just a few yodelling notes being enough to summon attention. Then, with luck, a Chowchilla will give a full performance in fine resonant tones: whooping, gobbling, yodelling – a rush of sound fit almost to shake leaves from the trees. The effect is hugely cheering and invigorating, the more so when other birds in the group add to the performance, inspired by the bird which initiated the singing. A listener can then move quietly forward and may be able to see a bird in full voice as it stands well up, chest and throat pumping strongly to fling the notes out, head jerking back and forth a little in emphasis . . . ."

❧❦

An excerpt from "Amongst Trees – Images from the Rainforests of North-east Queensland" (Russell et al. 2006)

Plate 1. Female Chowchilla, *Orthonyx spaldingii melasmenus*, engaging in territorial song at Mt. Lewis.

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Abstract

Intra-specific variation provides the basis on which evolutionary processes such as genetic drift, natural and sexual selection can act, creating distinct patterns of divergence within and among populations. Intra-specific variation and population divergence form the very beginning of the speciation process, and so determining the relative influence of different evolutionary processes in influencing current patterns of divergence is crucial in clarifying the mechanisms of the speciation process. Although most research focuses on patterns of genetic and morphological divergence, populations may also show divergence in cultural (learnt) behaviours such as song. Divergence in song is particularly intriguing, because it is crucially important in species and mate recognition as well as in sexual selection. In true songbirds (Oscines), songs are acquired from conspecifics though learning and imitation, defining bird song as a cultural trait that is non-genetically transmitted. Because song is learned culturally, inaccurate copying of the tutor song is possible and some level of spatial variation in song is inevitable. This, in turn, can lead to cultural evolution. Cultural and genetic evolution have many parallels and similarities, including the processes of mutation, drift, natural and sexual selection and cultural flow. Geographic variation in song could potentially influence population genetic divergence leading to speciation, by favouring within-dialect mating and natal philopatry, and discouraging between-dialect dispersal. Nevertheless, the notion that divergence in a cultural trait may promote speciation is still highly controversial.

Thus, the goals of this project were to determine the relative importance of different evolutionary forces in promoting geographic variation in song of the Chowchilla, *Orthonyx spaldingii* (Corvoidea), and to clarify the function and possible evolutionary consequences of such variation. In order to distinguish between the influence of different evolutionary forces, an integrative approach was used that combined patterns of genetic, and morphological variation with patterns of song variation. Therefore, the aims of my PhD were to (1) quantify the extent and pattern of large-scale geographic variation in song, morphology and neutral molecular genetic markers across the Chowchilla's entire range; (2) determine the evolutionary, cultural and social processes influencing both large and small-scale variation in song in order to clarify the functional significance of song variation; and (3) determine whether birds discriminate between local, distant and foreign song dialects to further establish the possible functions as well as evolutionary consequences (i.e. reduced gene flow or reproductive isolation) of geographic variation in song.

The Chowchilla is a rainforest specialist bird endemic to the montane tropical rainforests (the "Wet Tropics") of north-eastern Australia. The Chowchilla is remarkable for its striking, yet previously unstudied large-scale geographic variation in song, which is sung by both males and females alike in

territorial encounters. The Wet Tropics rainforests of Northern Australia experienced range contractions and expansions during the Pleistocene climatic fluctuations. As a consequence, many wet tropics endemic taxa are genetically divergent across an old climatic barrier, the Black Mountain Corridor (BMC), which has intermittently separated the northern and southern Wet Tropics. Limited evidence of morphological and molecular genetic divergence across the BMC also exists for the Chowchilla. Evidence of diversification in several character traits (molecular genetic, morphology and song) combined with the well established pattern of habitat expansion and contraction in the wet tropics, means that the Chowchilla provides an ideal and unique model system for comparing the evolutionary forces driving song variation with those thought to influence genetic and morphological divergence.

I recorded Chowchilla songs from 15 locations across the species' range, covering five historically isolated populations (Pleistocene refugia) and two areas of post-Pleistocene recolonisation. I measured six spectral characteristics of 773 songs and used a multivariate approach to test for large-scale song divergence within and among refugia, as well as across the BMC. Songs were also divided into their syllables, and a syllable catalogue was created for the whole population. Pair-wise comparisons of syllable sharing were then used to analyse song similarity at smaller spatial scales. I also collected blood samples and morphological measurements from 54 Chowchillas captured across their range. Morphological measurements were analysed using a Principal Components Analysis to determine the extent of morphological divergence among populations from different refugia, across the BMC, as well as between the sexes. Mitochondrial DNA (mtDNA) control region sequences from captured birds and museum specimens were analysed to determine the population genetic structure. Finally, I conducted playback experiments to determine whether Chowchillas differentiate between song variants from increasingly distant and isolated populations.

I found that historically isolated populations could be clearly distinguished by their spectrotemporal song characteristics, particularly bandwidth and peak frequency. I also found striking song divergence across the BMC. Northern refugia showed significantly narrower bandwidths and higher peak frequencies than southern refugia. Song characteristics were not influenced by geographic distance, habitat type or body size. Thus, given the known history of population isolation, song characteristics were most likely influenced by vicariant isolation in refugia followed by cultural drift. Chowchillas also showed exceptional small-scale variation in song syllable characteristics. Within historic refugia, song similarity was significantly correlated with distance. It was highest amongst neighbours and decreased sharply at one kilometre. These results are consistent with the idea of post-dispersal song learning, and reveal a strongly territorial function of song dialects.

Patterns of molecular genetic and morphological differentiation mirrored the pattern of large-scale song divergence across the Chowchilla's range. This suggests that historical isolation during

Pleistocene glaciation divided the Chowchilla into two distinct molecular lineages (northern and southern) that also have clearly divergent morphology and song characteristics. These findings demonstrate that vicariant isolation and genetic drift are sufficient to produce molecular genetic and phenotypic divergence in a rainforest specialist taxa. I also found significant and consistent sexual dimorphism in size and plumage colour in both northern and southern lineages, despite major size differences between lineages. This suggests that sexual selection as well as genetic drift have been important in shaping current patterns of morphological variation in both lineages. I also found no equivalent variation in bill morphology across the species entire range, suggesting that natural, stabilising selection associated with a specialized feeding niche may maintain bill characteristics in this species, independent of other morphological change.

Finally, playback experiments showed that Chowchillas recognised and approached their species-specific song in most playback trials irrespective of its origin. However, Chowchillas discriminated between local and foreign songs. Within lineages, groups sang significantly sooner and more often when hearing a local dialect *vs.* more distant song dialects. These results also support the territorial function of small-scale song variation. However, song of the alternative lineage elicited an unexpectedly high number of territorial responses. As it is highly likely that this territorial song also serves as a mate choice and advertisement function, recognition of foreign Chowchilla song means that song is unlikely to serve as a pre-mating barrier to gene flow in this species.

In conclusion, these results clearly show that all three evolutionary processes – genetic drift, natural and sexual selection – have concurrently influenced Chowchilla populations, creating contemporary patterns of divergence and variation in song, molecular genetic and morphological character traits, particularly across an old climatic barrier pre-dating the Pleistocene. Nevertheless, despite clear divergence in these traits, northern and southern populations have not diverged sufficiently to create a behavioural, pre-mating barrier to gene flow.

CHAPTER 1   Introduction

1.1   Variation and Genetic Evolution

Genetic variation is one of the fundamental pre-requisites of evolution (Ridley 1996). Variation within populations provides the foundation on which the evolutionary processes of genetic drift, sexual selection and/or natural selection can act. By redistributing variation, these processes create distinct patterns of divergence within and among populations. The forces of evolution thus shape population divergence and population divergence is the basis for speciation. Hence, understanding the evolutionary processes that promote population divergence is crucial to understanding the speciation process. Nevertheless, despite 150 years of evolutionary research since Darwin proposed the idea, speciation is still one of the least understood processes in evolutionary biology (Schluter 2001). Although the potential influence of each of the evolutionary processes is well established, their relative importance in promoting population divergence and ultimately speciation is not, leading to continuing controversy and debate amongst evolutionary biologists (Price 1998; Grant et al. 2000).

Intra-specific variation and population divergence form the very beginning of the speciation process, and so it is important not only to focus on the end point of speciation when two species have formed from one, but it is also crucially important to focus on the processes that initiate differentiation and divergence. Therefore, determining the evolutionary processes influencing current patterns of variation, their relative influence, as well as their combined effects, is crucial in clarifying the mechanisms of speciation.

1.2   Variation and Cultural Evolution

There is extensive empirical evidence that each evolutionary process can independently promote population divergence. Natural and sexual selection are particularly well-studied, as I will discuss below, with most previous research focussing on genetically determined phenotypic traits. Non-genetically based evolution may seem like a contradiction in terms. However, culturally transmitted traits can also evolve. This is because culturally transmitted traits fulfil the two requirements of evolution, i.e. variability and heritability in the units of change (Mundinger 1980; Cavalli-Sforza and Feldman 1981; Boyd and Richerson 1985; Lynch and Baker 1993).

The learning process that defines culture is thought to be an important and powerful creator of the variation that can lead to cultural evolution across generations (Dawkins 1976; Mundinger 1980; Cavalli-Sforza and Feldman 1981; Boyd and Richerson 1985; Lynch and Baker 1993; Lynch 1996). The concept of cultural evolution has attracted much scientific attention in the last two to three decades. One of the important realisations of this research is that cultural and genetic evolution have

many parallels and similarities, including processes of mutation, drift, natural and sexual selection and flow (Mundinger 1980; Cavalli-Sforza and Feldman 1981; Boyd and Richerson 1985; Baker and Jenkins 1987; Lynch et al. 1989; Lynch and Baker 1993; Lynch and Baker 1994; Payne 1996). However, whereas genetic evolution is precise and strictly vertical (transmission from parent to offspring), cultural evolution is prone to error because of the learning processes involved, and may be vertical, horizontal (between individuals within the same generation) or oblique (non-related individuals of different generations; Cavalli-Sforza and Feldman 1981; Lynch et al. 1989). Because of this, cultural evolution proceeds faster than genetic evolution and may be traced over shorter periods of time (Lynch *et al.* 1989). Furthermore, it is thought that cultural variation may influence genetic evolution, if divergence in cultural traits leads to assortative mating and/or natal philopatry, thereby reducing gene-flow between populations (see below).

1.3 Bird song as a cultural trait

Unlike most other avian taxa, in true songbirds (Oscines) as well as in hummingbirds (Trochilidae) and parrots (Psittacidae), songs are acquired from conspecifics though learning and imitation, usually very early in a bird's life (Beecher and Brenowitz 2005). This learning process defines bird song as a cultural trait that is non-genetically transmitted (Mundinger 1980; Baker and Cunningham 1985). Because song is learned culturally, inaccurate copying of the tutor song is possible and some level of spatial variation in song is inevitable (Slater 1989).

Divergence in acoustic communication behaviour such as bird song is particularly intriguing, because bird song is crucially important in species and mate recognition as well as in sexual selection (Catchpole and Slater 1995; Price 1998). Therefore, it potentially plays a major role in bird speciation and diversification theory (Martens 1996; Price 1998). Due to its important functions in social and reproductive interactions bird song should, theoretically, be under strong stabilising selection (Martens 1996). In other words, if the song of an individual diverges too far from its species-specific template the consequences in terms of fitness could potentially be dire, including social exclusion and reproductive failure (Martens 1996). Nevertheless, geographic variation in song is a common phenomenon among the songbirds (Catchpole and Slater 1995; Martens 1996) and leads to intriguing questions such as: What processes promote song divergence between populations? How is song divergence between populations maintained? What is the functional significance of song variation/divergence? What are the evolutionary consequences of song divergence, i.e. can divergence in song influence genetic divergence and, ultimately, speciation?

Song is a mating signal and, thus, plays a pivotal role in social and reproductive interactions in birds. Divergence in mating signals is thought to be a strong driver of speciation, particularly because differences in mating signals are consistently found to be powerful isolating mechanisms between species (Martens 1996). Consequently, geographic variation in song could potentially influence

population genetic divergence leading to speciation, by favouring within-dialect mating and natal philopatry, and discouraging between-dialect dispersal (Irwin and Price 1999; Slabbekoorn and Smith 2002a). Nevertheless, the notion that divergence in a cultural trait may promote speciation is still highly controversial (Grant and Grant 1997b; Irwin and Price 1999; Slabbekoorn and Smith 2002a).

Darwin's finches on the Galapagos Islands are a classic example where song divergence in geographically isolated populations has resulted in a behavioural pre-mating barrier to gene flow on secondary contact (Grant and Grant 1997b; Grant and Grant 1997a). Four species of Ground finch live sympatrically on Daphne Major island. Hybridisation occurs between three of the species (*Geospiza fortis*, *G. scadens* and *G. fuliginosa*), and viable hybrids can be produced. However, levels of hybridisation are very low due to the fact that each species will only mate with birds that sing their own (species-specific) song. Hybridisation only occurs after young birds misimprint on heterospecific song rather than their own (Grant and Grant 1997b; Grant and Grant 1997a). Thus, divergence in song can indeed act as a behavioural pre-mating barrier to gene flow that may ultimately cause speciation. The potential influence of cultural divergence on the speciation process will be further discussed in Chapter 2.

1.3.1 Natural selection

Natural selection has received much attention and has been shown to promote divergence in many characters (Ridley 1996; Schluter 1996; Grant 1999; Schluter 2001; McKinnon and Rundle 2002; Slabbekoorn and Smith 2002b). As with bird song, one particularly well-studied system and a classic example of diversification through natural selection is beak morphology in Darwin's finches (Grant 1999; Grant and Grant 2002b). In addition, the strong influence of natural selection on morphological divergence has also been clearly shown in Little greenbuls, *Andropadus virens* (Smith *et al.* 1997).

The influence of natural selection on divergence in signalling behaviour has also been demonstrated in several species of songbird. Differences in habitat structure can influence song characteristics due to differential sound attenuation and degradation in different habitats. Birds occupying open habitats tend to sing at higher song frequencies with more frequency modulations and trills compared to birds living in closed habitats (Wiley 1991; Badyaev and Leaf 1997). Thus, song characteristics of different populations may diverge as a result of natural selection maximising transmission through different habitat types (acoustic adaptation hypothesis; Morton 1975). Natural selection can also indirectly influence song characteristics through changes in morphology, particularly body size and beak morphology. Larger body size can result in lower song frequencies and narrower song bandwidths (Ryan and Brenowitz 1985; Wiley 1991; Badyaev and Leaf 1997; Doutrelant et al. 2001; Nowicki et al. 2001), while larger beaks can also be associated with lower trill rates and lower frequencies (Palacios and Tubaro 2000; Ballentine 2006; Huber and Podos 2006).

1.3.2 Sexual selection

The influence of sexual selection on morphological and behavioural divergence has also received considerable attention (e.g. Bateson 1983; Andersson 1994; Panhuis et al. 2001; Andersson and Simmons 2006). Intrasexual competition linked to mate choice and sexual selection has been shown to produce sexual dimorphism, weaponry, conspicuous colouration and/or elaborate song, usually in males (Hill 1991; Andersson 1994; Owens and Hartley 1998; Hadfield et al. 2006). In addition, research has consistently shown that female birds prefer males with longer, more complex songs and/or larger song repertoires, presumably because the ability to sing such songs is an honest indicator of male quality (Ryan and Keddyhector 1992; Price 1998; Slater 2003). Thus, while it is clear from these studies that sexual selection can create and maintain intra-specific variation in traits used for mate choice, its importance in promoting speciation is still debated (Price 1998; Slabbekoorn and Smith 2002a).

1.3.3 Genetic & cultural drift

Theoretically, population divergence may also arise as a result of random microevolutionary processes (genetic drift; Futuyma 1998). However, direct empirical evidence for population divergence having arisen by genetic drift alone remains scarce. Genetic drift has been used to explain molecular genetic, morphological and behavioural differences, particularly among populations isolated on islands and/or between island and mainland populations, when the influence of other evolutionary processes is unlikely (Martens 1996; Mundy et al. 1997; Price 1998; Clegg et al. 2002a; Clegg et al. 2002b; Knowles and Richards 2005; Baker et al. 2006). For example, drift is thought to have affected levels of divergence in island populations of Silvereyes, *Zosterops lateralis lateralis* (Clegg *et al.* 2002a), as well as in a newly established island-population of Large Ground finches, *Geospiza magnirostris* (Grant and Grant 2001).

Similar to genetic drift, cultural drift in song is the random change in the frequency of song elements or song types across generations. Such cultural drift has been shown in several island/mainland songbird populations. For instance, Singing honeyeaters, *Lichenostomus virescens*, showed a reduced song element pool and lower syllable diversity in an island population compared to mainland populations. This reduction was attributed to founder effects at the time the island was colonised (Baker *et al.* 2001). Vicariant isolation followed by drift was also used to explain geographic divergence in spectral song characteristics of the Golden bowerbird, *Prionodura newtonia* (Westcott and Kroon 2002).

The examples given in the preceding paragraphs demonstrate that each evolutionary mechanism can influence current patterns of morphological, genetic and behavioural divergence independently. They also show that in order to gain a better understanding of the relative importance of the different evolutionary processes underlying divergence, it is crucial to establish the pattern and extent of intra-

specific variation in a range of character traits. The study of the evolutionary processes promoting and maintaining song divergence also provides a fascinating, non-genetic counterpart to the processes creating genetic and morphological divergence.

1.4　The study system

I investigated the relative influence of different evolutionary processes in creating patterns of song, molecular genetic and morphological divergence in the Chowchilla, *Orthonyx spaldingii* (Corvoidea), an endemic songbird that occurs only in the upland rainforests of north-eastern Australia (the 'Wet Tropics') between the Finnegan Uplands south of Cooktown (15°30′S, 146°16′E) and the Paluma Range north-west of Townsville (19°15′S, 146°45′E). The Chowchilla is a ground-dwelling insectivore that lives in small groups of two to six birds that cooperatively defend stable territories of up to two hectares (Jansen 1999). All members of a group, including females, sing a very loud and conspicuous territorial song (henceforth simply called song), which is sung year-round during the dawn chorus and during the day in occasional territorial encounters with neighbours.

This species is particularly suited for a study of the forces driving geographic variation in song as exceptional geographic variation in song at larger spatial scales has been suggested (Westcott, pers. com.), it shows a mosaic distribution of song dialects at smaller scales (McGuire 1996), and unlike most songbirds it has a single-song repertoire with both males and females singing identical territorial song. Despite its loud and charismatic territorial song, geographic variation has not been quantified previously across the range of this species.

The well-documented historical effects of climate variation on the distribution of Wet Tropics rainforest vegetation also means that endemic songbirds of the Wet Tropics are particularly useful for studying the forces influencing intra-specific variation and divergence. The rainforests of the Wet Tropics World Heritage Area have undergone major range contractions and subsequent expansions due to climatic oscillations since the late Tertiary (2.4 million years before present, bp; Webb and Bartlein 1992; Hewitt 1996). These climatic fluctuations and their impact on rainforest distribution are particularly well-documented for the most recent glacial cycle, which began approximately 135 000 years bp and reached its glacial maximum 18 000 years bp (Webb and Bartlein 1992; Hopkins et al. 1993; Kershaw 1994). At the height of the last glaciation, the Wet Tropics rainforests were restricted to a number of small, montane rainforest refugia separated by climatic barriers unsuitable for rainforest (Webb and Tracey 1981; Nix 1991; Fig. 1.1a). With the advance of more favourable climate around 7 000 years bp, the rainforests expanded again, resulting in the current extent of relatively continuous rainforests (Fig. 1.1b).

Based on pollen records and the distribution of primitive angiosperms, Webb & Tracey (1981) proposed a number of small Pleistocene refugia throughout the Wet Tropics. Alternatively, Nix and

Switzer (1991) proposed two larger isolates of suitable rainfall based on climatic modelling, which overlap many of Webb & Tracy's (1981) refugia. Both Nix and Switzer's (1991) isolates and Webb & Tracey's (1981) refugia are divided by a much older, climatic barrier known as the 'Black Mountain Corridor' (BMC), separating the proposed remnants into northern and southern isolates/refugia (Webb and Tracey 1981; Nix and Switzer 1991; Hopkins et al. 1993). This history of contraction into isolated refugia and subsequent expansion of the rainforest has also affected the genetic structure of populations inhabiting these refugia. Many high-altitude, rainforest restricted Wet Tropics endemics show a remarkably congruent pattern of strong mtDNA divergence across the BMC, as well as patterns of expansions and (re)colonisation within the northern and southern isolates (Joseph et al. 1995; Winter 1997; Schneider et al. 1998; Schneider and Moritz 1999; Schneider et al. 1999; Hugall et al. 2002). These patterns will be further discussed in Chapter 5.

The Chowchilla would also have experienced strong population fluctuations during the Pleistocene. Limited genetic and morphological evidence suggests divergence across the BMC in a number of character traits (Joseph et al. 1995; Schodde and Mason 1999), separating the species into two sub-species, *O. s. spaldingii* south of the BMC, and *O. s. melasmenus* north of the BMC (Schodde and Mason 1999). This preliminary evidence of divergence, in addition to evidence of geographic song variation at several scales and the well-established history of refugial isolation, provides an ideal natural experimental design, which allows for a unique comparison between the forces driving song variation and those thought to influence genetic and morphological divergence.
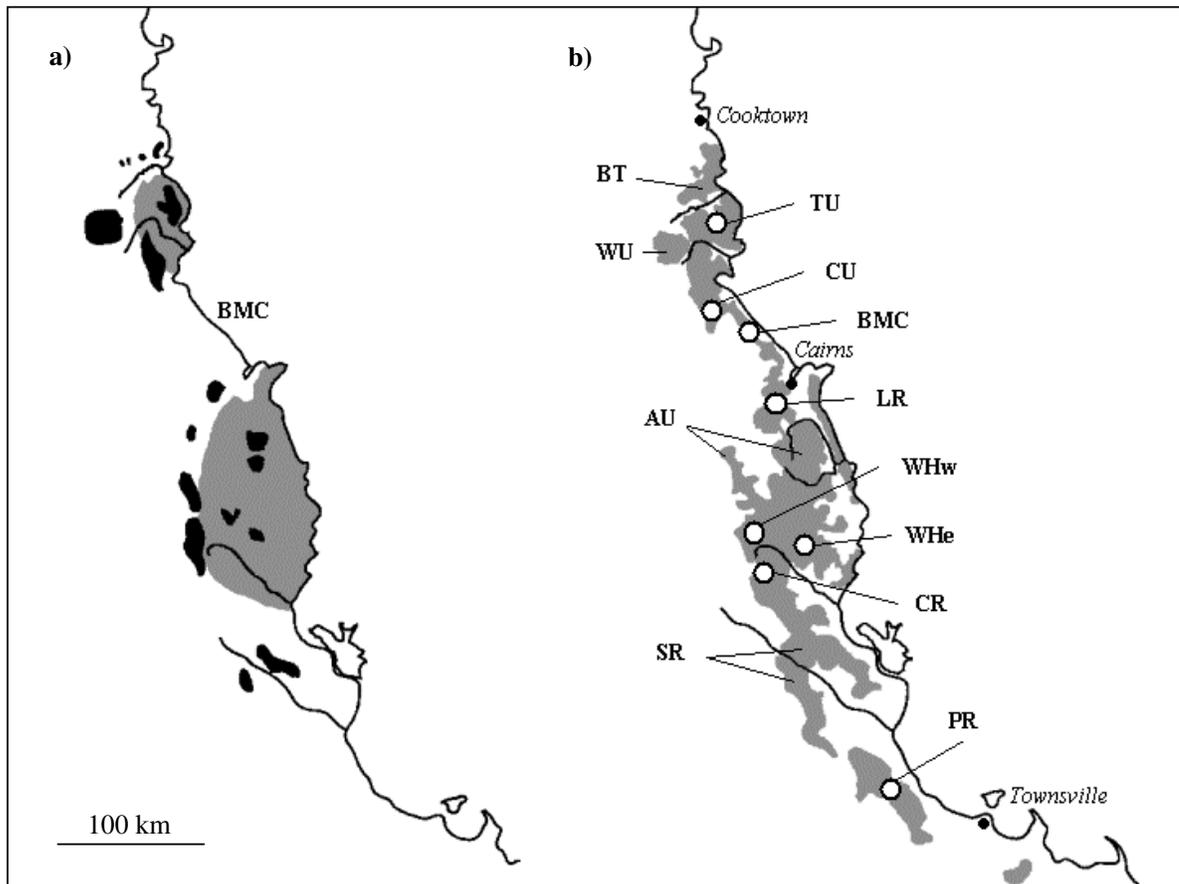
Figure 1.1. Map showing a) Pleistocene refugia as proposed by Webb & Tracey (1981; black shading), and northern/southern isolates as proposed by Nix & Switzer (1991; grey shading); and b) locations of sampling sites (white circles) across the current Wet Tropics rainforests (grey shading), in north-eastern Australia. BT – Big Tablelands, TU – Thornton Uplands, WU – Windsor Uplands, CU – Carbine Uplands, BMC – Black Mountain Corridor, LR – Lamb Range, AU – Atherton Uplands, WH – Walter Hill Ranges (w – west & e – east), CR – Cardwell Range, SR – Seaview Range, PR – Paluma Range; BMC and PR represent areas of post-Pleistocene recolonisation.

1.5   Aims and thesis structure

The goals of this project were to determine the relative importance of different evolutionary forces in promoting geographic variation in song of the Chowchilla, and to clarify the function and possible consequences of such variation. In order to distinguish between the influence of the different evolutionary forces, an integrative approach was used that undertook comparative analysis among patterns of genetic, morphological and acoustic variation over a range of spatial scales.

Therefore, the aims of my PhD were to:

1)   quantify the extent and pattern of large-scale geographic variation in song, morphology and a
     neutral molecular genetic marker across the Chowchilla's range;

2) determine the evolutionary, cultural and social processes influencing small-scale variation in song in order to clarify the functional significance of local song variation; and

3) determine whether birds discriminate between local and foreign song dialects to further establish the possible functions as well as evolutionary consequences (i.e. reduced gene flow or reproductive isolation) of geographic variation in song.

The thesis is divided into six chapters. Chapter 1 introduced the general background of this research and the aims and structure of this thesis. Chapter 2 explores and quantifies large-scale song variation in the Chowchilla and identifies the most likely evolutionary processes creating such variation. In Chapter 3, the patterns of small-scale variation in song are described and analysed, to determine the functions of local song variation. Chapter 4 then examines the influence of different evolutionary processes on morphological as well as molecular genetic divergence. Chapter 5 describes song playback experiments and analyses to further investigate the function of song variation as well as the evolutionary consequences of such variation. The overall findings of this project are then synthesised and summarized in Chapter 6, including a general discussion of the overall implications of this research and suggestions for future research.

CHAPTER 2   Large-scale Song Divergence

2.1   Introduction

Large-scale processes thought to influence patterns of song divergence are similar to those driving genetic divergence: vicariant isolation followed by drift and/or selection of certain song types or song characteristics, and isolation over geographic distances (isolation-by-distance) resulting in the accumulation of small differences as distant individuals are less likely to socially interact (Cavalli-Sforza 2000). Under the vicariance and drift model, song variation would be expected to be greatest between populations that have been isolated the longest, irrespective of the distance between them. Thus, song is expected to be more similar within than among isolated populations, even when distances within the population are larger than among populations. On the other hand, under a drift only model, song similarity would be expected to decrease linearly with distance due to the accumulation of small differences across space (isolation-by-distance).

The influence of isolation and subsequent drift on song has been shown in some species that occur both in mainland populations as well as in island populations that established through a founder-event. The initial bottle-neck effect in small founder populations can lead to a reduced number of song types or song elements due to random sampling effects. Once isolated, subsequent drift may lead to further simplification or modification of the song (Mundinger 1980; Baker and Jenkins 1987; Grant and Grant 1995; Baker 1996; Slabbekoorn and Smith 2002a, b; Baker et al. 2003a; Baker et al. 2003b; Podos et al. 2004a). Vicariant isolation and founder effects followed by drift were used to explain island/mainland song divergence in Singing honeyeaters, *Meliphaga virescens* (Baker *et al.* 2001), as well as Golden bowerbirds, *Prionodura newtonia* (Westcott and Kroon 2002).

Alternatively, song characteristics may vary geographically due to the acoustic properties of different habitats, or due to different ecological conditions (Slabbekoorn and Smith 2002a, b; Ruegg et al. 2006). Under this acoustic-adaptation model, song characteristics would be strongly linked to differences in ecology or habitat, but not with geographic location or distance. Large-scale song variation was clearly linked to habitat differences in the Satin bowerbird, *Ptilonorhynchus violaceus*, as call structure converged in similar habitats but diverged among different habitats independent of geographic location or distance (Nicholls and Goldizen 2006). Similarly, Ruegg *et al.* (2006) showed that song characteristics were strongly correlated with ecological (climatic) variables after correcting for genetic distance in Swainson's thrush, *Catharus ustulatus*. Baker (2006) also found a change in

spectral characteristics with changing ambient sound environments between mainland and island populations of both Red capped robins, *Petroica goodenovii,* and Western gerygones, *Gerygone fusca*.

Finally, divergence in song may also be indirectly influenced by changes in morphology, particularly body size and beak morphology. Peak or dominant frequency have been shown to be affected by body size, and organisms with larger body sizes generally produce sounds of lower dominant or peak frequencies and narrower bandwidths (Ryan and Brenowitz 1985; Wiley 1991; Badyaev and Leaf 1997; Doutrelant et al. 2001). In addition, it has been suggested that beak size can also constrain song production. It has been shown in some species that birds with larger beaks produce songs of lower minimum and maximum frequencies as well as narrower bandwidths (e.g. Woodcreepers, Palacios and Tubaro 2000; e.g. Darwin's finches, Podos et al. 2004b; Huber and Podos 2006).

Despite the growing number of studies focussing on geographic variation in bird song, the relative importance and strength of the different forces in influencing song variation and divergence have still not been resolved unambiguously. Few studies have systematically recorded song across the entire range of a species to establish the pattern and extent of geographic song variation in order to test the different theories of the origin of song variation.

As outlined in Chapter 1, preliminary evidence of geographic song variation in Chowchilla song at several spatial scales as well as the well-established history of refugial isolation in the Wet Tropics provides an ideal natural experimental design to test whether historical population isolation contributed to current landscape-scale geographic variation in song in this species.

Therefore, the aims of this Chapter are to:
1) explore and quantify the extent and pattern of current, geographic variation in song across the Chowchilla's entire range;
2) determine whether this spatial pattern in song variation can be explained by the influence of vicariant isolation, isolation-by-distance, vegetation type and/or body size and mass.

Thus, under the vicariance and drift model, clear differences in song characteristics would be expected between historically isolated refugia and particularly across the older BMC irrespective of the distance between them. On the other hand, if geographic distance rather than vicariant isolation contributed to current song variation, a significant, positive relationship of song dissimilarity with distance within refugia would be expected. If habitat type influenced song characteristics, clear song divergence between different habitat types and convergence of song characteristics in similar habitat types would be expected, irrespective of location or distance. Finally, if body size or beak morphology affected song divergence in the Chowchilla, a significant, negative relationship between song frequency and body size and/or beak size would be expected.

2.2 Methods

2.2.1 Study Sites

Recordings of Chowchilla song were made at 15 locations across the species range, covering five Pleistocene refugia as proposed by Webb & Tracey (1981; see Fig. 1.1a & b): Thornton Uplands (TU), Carbine Uplands (CU), Lamb Range (LR), Walter Hill Ranges (WH), and Cardwell Range (CR). In addition, song was recorded from two areas of post-glacial recolonisation: the Black Mountain Corridor (BMC) and the Paluma Range (PR). Latitudes and longitudes of all locations are given in Table 2.1. Songs were recorded between April and November 2004 and August and November 2005.

Geographic variation was sampled at different spatial scales that form a hierarchical, or nested, order of scale. At the largest scale, Chowchilla populations are divided into northern and southern isolates either side of the BMC (see Fig. 1.1a & b). Within these northern and southern isolates, several of the suggested Pleistocene refugia (Webb and Tracey 1981) were sampled in this study. Within each refuge, I visited one to six different locations, and at each of these locations, I recorded Chowchilla vocalizations at one to two sites. At each of these sites, I recorded songs from as many groups of Chowchillas as possible during the dawn chorus (2-15 groups per site, average number of groups per site $\pm$ SD = 6.2 $\pm$ 4.1). Thus, I sampled at decreasing scales from the largest scale (northern and southern populations divided by the BMC), to medium scales (refugia), to the smallest scale (locations and sites; Table 2.2).

Table 2.1. Latitudes and longitudes of recording locations within each refuge and area of recolonisation. Number of sites within Lamb Range locations shown in brackets.

Refugia	Locations (no. of sites)	Latitude	Longitude
Thornton Uplands, TU	Creb track (1)	S16°06.0596'	E145°20.2002'
Carbine Uplands, CU	Mt. Lewis 1 (2)	S16°35.1612'	E145°16.1331'
	Mt. Lewis 2 (1)	S16°32.2471'	E145°17.1416'
Black Mountain Corridor, BMC*	Black Mountain (2)	S16°36.6600'	E145°27.3782'
Lamb Range, LR	Douglas Track (1)	S16°52.9813'	E145°37.9718'
	Clohesy Fig (2)	S16°56.2495'	E145°36.9415'
	Clohesy Rd (1)	S16°58.6937'	E145°39.0068'
	Lake Morris (2)	S16°58.6222'	E145°41.7588'
	Davies Ck (2)	S17°02.3359'	E145°36.7328'
	Kauri Ck (3)	S17°07.6920'	E145°36.3283'
Walter Hill Ranges, WH	South Johnstone (2)	S17°38.4473'	E145°42.8053'
	Misty Mountains (2)	S17°41.1763'	E145°31.3067'
Cardwell Range, CR	Koombooloomba Dam (2)	S17°50.3819'	E145°35.6926'
Paluma Range, PR*	Paluma Dam (2)	S18°57.6036'	E146°08.8720'
	Paluma town (2)	S19°00.4985'	E146°12.6544'

* areas of post-glacial recolonisation

Table 2.2. Average distances (km) ± 2SE between sampling refuges, locations and sites. The number of between site-comparisons (*N*) from which the data was derived is given.

	N	Mean distance ± 2SE
Between refuges N/S of BMC	26	151.2 ± 42.1
Between refugia (S only)	156	106.4 ± 12.8
Between refugia (N only)	2	54.6 n/a
Between locations within refugia	36	12.8 ± 2.2
Between sites within locations	65	2.7 ± 0.3
Within sites	90	0.15 ± 0.04

2.2.2 Song Recordings

At each location, Chowchilla vocalizations were recorded at two sites between one and four kilometres apart. At each site, songs of as many independent groups of Chowchillas as possible were recorded. Chowchillas started singing at dawn and continued for about 30-60 minutes (mins). During this time, individual groups of Chowchillas were recorded for 2-5 mins each, or a minimum of 10 songs. This resulted in transects of 500-1000 meters (m) in length at each site. All groups recorded at distances less than 1000 m were assigned to the same site. Each group's position along the transect was recorded.

During the morning chorus, all Chowchilla groups in an area sing at the same time with members of each group taking turns. Chowchillas tend to sing from their roosting trees within their territories before descending to the ground (Jansen 1993), and group members tend to be very close to one another (personal observation). Therefore, songs from single groups could be confidently located and identified, but the recognition of individual birds in each group was not possible. Although the exact number of birds per group was unknown, it was clear that in all cases there were at least two birds in a group, singing identical songs. At some sites, Chowchillas sang for too short a time, or they were too far away for good-quality recordings during the dawn chorus. In these instances, playback was successfully used to tempt the birds into approaching and singing in order to record vocalizations of better quality. Chowchillas did not alter their song when responding to playback compared to the song used during dawn chorus (personal observation).

Songs were recorded from as close to the groups as possible using a Tascam DA-P1 digital audio tape recorder and a Sennheiser ME-67 directional microphone. Recordings were downloaded onto a Toshiba notebook (Toshiba Tecra Intel Celeron M) and digitised using a Sigma Tel Audio 5.10 soundcard at 22 050 Hz sampling frequency with 16-bit precision. Recordings from each morning were stored as individual sound files. Songs were analysed using Avisoft-SASLab Pro (Version 4.38, Specht 2005). Spectrograms for each song were produced with a 512-pt Fast Fourier Transform

(frequency resolution 43 Hz, time resolution 2.90 milliseconds, Flat Top window and 87.5% overlap). Each song was given an identifying code.

Up to 10 best-quality songs per group (average number of songs per group $\pm$ SD = 7.89 $\pm$ 3.6) were chosen by visually assessing quality on the spectrograms, selecting only those songs showing low background noise, low distortion of songs and elements, and high differentiation of the sound trace from background noise on the spectrogram. All songs were subjected to a low-pass band filter set at 0.60 kHz to eliminate any low frequency background noise.

2.2.3 Spectrotemporal Song Measurements

Temporal and spectral measurements of each song were recorded at the mean spectrum of each whole song (i.e. measurements are derived from the averaged spectrum of the entire element or song; Specht 2005), using the automatic parameter measurement tool in Avisoft SASLab Pro (Specht 2005). Parameters measured included song length (seconds, secs), peak frequency (the frequency at the maximum amplitude, Hz), minimum frequency (Hz), and bandwidth (the difference between maximum and minimum frequency, Hz; Fig. 2.1). In addition, the number of distinct elements within each song was counted from the spectrograms and used to calculate the element rate (number of elements divided by song length; Fig. 2.1; see also Section 3.2.3 for a clear definition of elements and syllables).

To compare overall spectral similarity between locations and to reduce the dimensionality of the data, non-metric multidimensional scaling (NMS) was performed on the six variables described above. NMS is an ordination technique based on ranked distances and thus is better suited for data that are non-normal or for data of different scales (Clarke 1993; McCune and Mefford 1999). As the song data was non-normally distributed and, hence, violated the assumptions of more commonly used multivariate approaches (such as Principal Components Analysis), NMS was deemed more appropriate. A two-dimensional solution yielded the least stress (stress = 0.966) and thus was most suitable when arranging the data points in multivariate space. The correlation coefficients (loadings) were used to determine which two variables described most of the variation between locations. A multi-response permutation procedure (MRPP) was then used to test for multivariate differences among the a priori grouping variables (refuge and location). MRPP is a non-parametric procedure for testing the hypothesis of no difference between two or more groups of entities, and it also does not require adherence to assumptions such as normality (Zimmerman et al. 1985; Mielke and Berry 1994; McCune and Mefford 1999).

For clarification of the patterns observed, averaged NMS scores ($\pm$ 2 S.E.) for each refuge were plotted separately for both dimensions and compared using one-way ANOVAs and Tukey HSD post hoc tests.
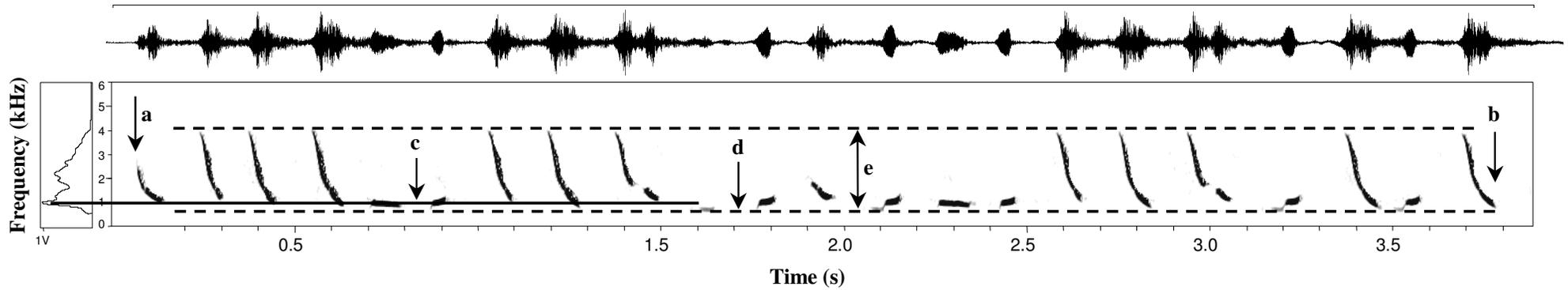
Figure 2.1. Song spectrogram of a Chowchilla song from the Lamb Range (Davies Creek dialect), showing the song's waveform (upper figure) and power spectrum (left figure); each black trace on the spectrogram itself denotes one element, which was counted to determine the number of elements and the element rate of that song; the frequency parameters used for further analysis were measured at the mean spectrum of the whole song using Avisoft SASLab Pro (Specht 2005) and are highlighted within the spectrogram: (a) start of song, (b) end of song, (c) peak frequency (frequency with the most energy as shown on power spectrum, Hz), (d) minimum frequency (Hz), (e) bandwidth (difference of maximum and minimum frequency, Hz).

2.2.3.1   Effect of vegetation type

In order to test for the effects of different habitat types on song spectrotemporal variables, I averaged NMS scores for each site and identified each site's vegetation type following Tracey's (1982) vegetation classification. Habitat types observed at my study sites included 'Complex Mesophyll Vine Forest' (Type 1 a & b; CMVF), 'Mesophyll Vine Forest' (Type 2a; MVF), 'Complex Notophyll Vine Forest' (Type 6; CNVF), and 'Simple Notophyll Vine Forest' (Type 8; SNVF). Vegetation types that only occurred at one site (Type 6) were excluded. To detect any patterns or clustering of vegetation types, site-averaged NMS scores were plotted in multidimensional space using vegetation identifiers for each site. If vegetation type had an effect on song characteristics (NMS scores), I would expect a clustering of sites according to vegetation type.

2.2.3.2   Effect of geographic distance

In order to determine the influence of geographic distance on spectrotemporal differences, pair-wise song differences between all locations were calculated by computing squared Euclidean distances (dissimilarities) from each location's NMS scores. Pair-wise geographic distances (km) between all locations were determined using the Route function in a GPS unit (Garmin *etrex* Summit Version 2.06, Garmin Corp., Olathe, KS, U.S.A.). To test whether spectrotemporal differences were correlated with distance, I used the non-parametric Mantel randomisation test, which evaluates the null hypothesis of no relationship between two similarity or dissimilarity matrices (Manly 1997). The Mantel test is an alternative to regressing distance matrices that avoids the problem of partial dependence in the matrices (Manly 1997). The Mantel test statistic *r* was calculated using a Monte Carlo randomisation procedure with 5000 permutations in PC-ORD (Version 4; McCune and Mefford 1999).

2.2.3.3   Effect of body size

Fifty-three Chowchillas were captured (25 females, 28 males) at 11 locations across their range ($n = 2$-9 birds per location, average number of birds per location = 4.6), covering all five refugia and two areas of recolonisation. For each captured bird, I measured a number of standard morphometric measurements, including body mass (g), bill length and tarsus length (mm). Full details of the method of capture and morphological measurements can be found in Chapter 4. Birds were sexed by their plumage and all captured birds were adults.

To detect a possible effect of body size on song frequency, body mass, tarsus length and bill length were averaged for each location and compared to the average peak frequency and average bandwidth used at that location. As it was not possible to capture Chowchillas at every song recording site (due to time constraints as well as particularly clever birds), song data used for this analysis only includes songs recorded at locations where Chowchillas were also captured. In addition, due to previously suggested divergence in body size across the BMC (Schodde and Mason 1999; see also Chapter 4)

only southern locations were used in this analysis ($n = 8$ locations), in order to avoid the possible confounding effect of body size variation across the BMC. However, data for the northern locations were included in the graphs for comparison. The relationships between body size/mass and bill length *vs.* peak frequency and bandwidth were tested using linear regression analyses, after *log*-transforming any morphological characters that did not adhere to normality (normality was assessed using Shapiro-Wilk's test).

All statistical analyses were performed using SPSS 13.0 for Windows (SPSS 2003) and PC-Ord for Windows (McCune and Mefford 1999) unless otherwise stated. Significance levels were set at $\alpha < 0.05$. All results are given as average $X \pm 2$ SE unless otherwise stated.

2.3 Results

2.3.1 Chowchilla song

During territorial encounters, group members took turns singing, alternating between the two or more groups involved in the conflict. Individual birds occasionally cut their song short when disturbed or when interrupted by another singing Chowchilla (from their own or a different group). Chowchillas also increased song length by continued singing of the whole song, or by repeating the latter half of their song. However, within a specific cooperative group the basic single song type was defined by a certain series of syllables or elements that did not change over the period of this study.

Songs differed qualitatively in the number and type of elements and broad frequency range (Fig. 2.2). Chowchilla song was made up of a fixed sequence of pure notes, and songs were characterized by a diversity of different notes. Birds in a locality repeated the same sequence of notes with each song unless the song was cut short. Song of the southern populations (LR, WH, CR and PR) were characterized by the prevalence of descending notes of broad bandwidths (A-notes; Fig. 2.2d-g; see also Chapter 3, Fig. 3.3), interspersed by shorter notes that were descending, ascending or without a change in pitch (B, C and W-notes). The song of most southern populations started with a short descending note followed by a repetition of two to four A-notes (Fig. 2.2d-g). Songs of the northern populations (TU and CU) were made up of similar notes but of narrower bandwidths, especially the A-notes. Songs were dominated by short notes with only some A-notes interspersed (Fig. 2.2a & b). The occurrence of unique notes (D-notes) was common to all populations. For a more detailed description of the different notes please refer to Chapter 3.

A total of 773 songs from 93 groups of Chowchillas were chosen for the analyses (average of 8 songs per group and 6 groups per site). Average duration, peak frequency, minimum frequency and bandwidth $\pm$ SD are 4.5 $\pm$ 1.7 secs, 1523.8 $\pm$ 448.8 Hz, 809.5 $\pm$ 128.2 Hz and 2515.6 $\pm$ 411.8 Hz, respectively ($n =$ 773; Table 2.3). Notable was the difference in bandwidths between northern and

southern refugia; northern refugia showed bandwidths below 2000 Hz whereas southern refugia showed bandwidths clearly above 2500 Hz (Table 2.3).

Table 2.3. Average spectrotemporal measurements ± SD for 773 Chowchilla songs recorded in five refugia (TU, CU, LR, WH, CR) and two areas of post-glacial recolonisation (BMC, PR) in the Wet Tropics, Australia. Spectrotemporal measurements include duration (secs), peak frequency, minimum frequency and bandwidth (Hz).

Refugia	Duration ± SD	Peak Frequency ± SD	Minimum frequency ± SD	Bandwidth ± SD
TU	3.50 ± 1.27	1496.36 ± 401.44	983.18 ± 100.94	1535.68 ± 110.76
CU	6.81 ± 3.32	1470.50 ± 319.79	849.83 ± 141.56	1921.00 ± 191.87
BMC*	3.30 ± 0.84	1857.96 ± 537.40	803.67 ± 102.46	2097.76 ± 121.30
LR	4.29 ± 1.43	1600.49 ± 439.27	758.66 ± 148.44	2671.37 ± 276.99
WH	4.17 ± 1.13	1360.00 ± 317.82	826.58 ± 79.53	2623.78 ± 258.63
CR	4.23 ± 1.20	1514.14 ± 468.29	820.61 ± 77.91	2671.11 ± 221.54
PR*	5.00 ± 0.99	1407.94 ± 485.48	822.94 ± 80.54	2734.79 ± 199.11
Total	4.47 ± 1.70	1523.82 ± 448.84	809.53 ± 128.21	2515.59 ± 411.77

*areas of post-glacial recolonisation
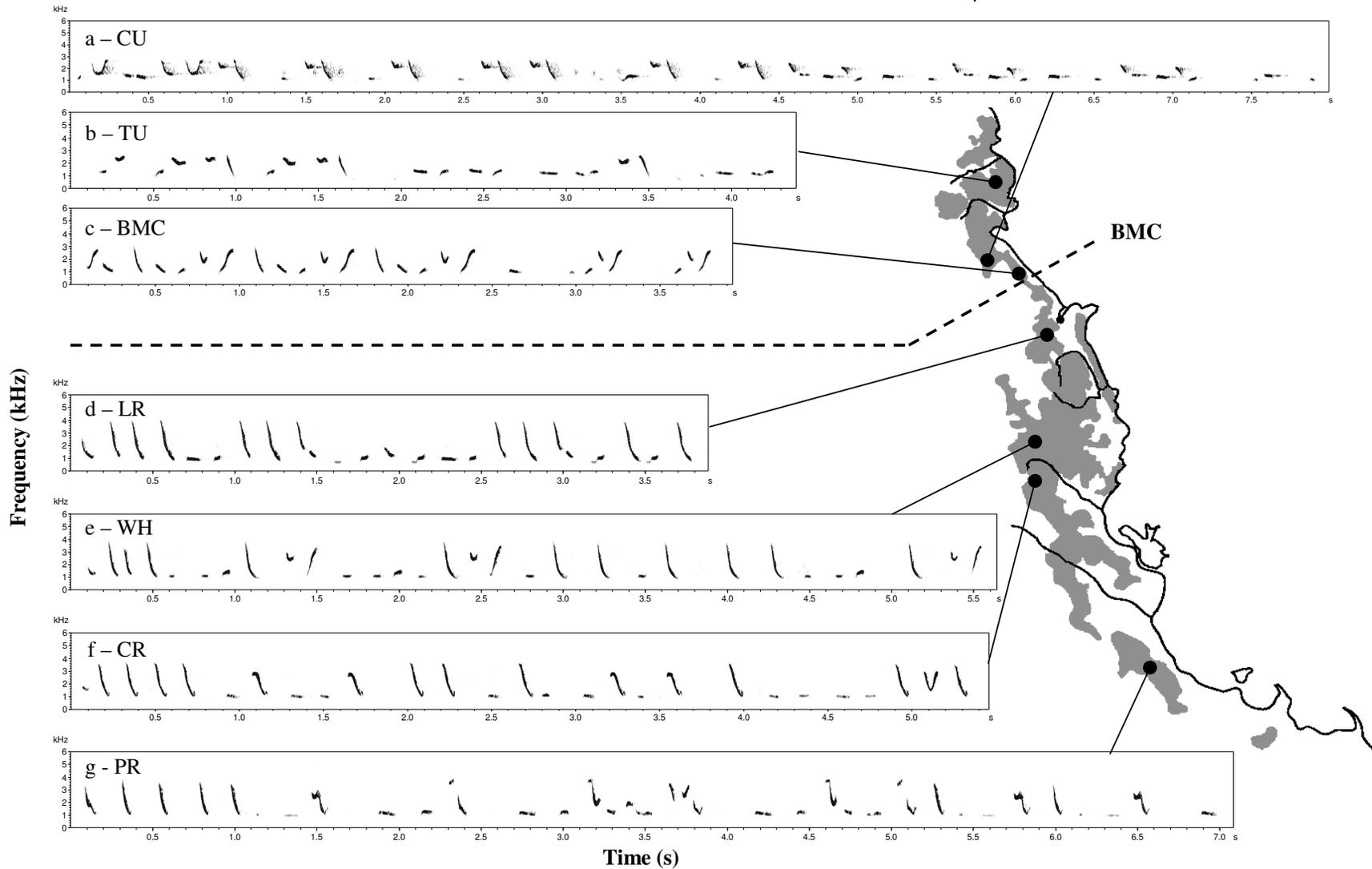
Figure 2.2. Example spectrograms of seven Chowchilla songs from five different refugia and two areas of recolonisation (a – TU; b – CU; c – BMC; d – LR; e – WH; f – CR; g - PR) within the Wet Tropics of Australia. Position of the Black Mountain Corridor (BMC) indicated by dashed line.

2.3.2 Spectrotemporal Song Measurements

Non-metric multidimensional scaling of the six spectrotemporal song variables showed that variation across Axis 1 (Dimension 1) was mainly influenced by differences in bandwidth, whereas variation across Axis 2 (Dimension 2) was mainly explained by differences in peak frequency (Table 2.3). Higher Dimension 1 values corresponded to wider bandwidths, whereas higher Dimension 2 values were associated with lower peak frequencies (Table 2.3).

When plotting average NMS scores for each refuge in multidimensional space, there is a strong separation between northern *vs.* southern refugia, with song from the northern refugia (including the BMC) having narrower bandwidths and higher peak frequencies than the southern refugia (Figs 2.3 & 2.4). Southern refugia are characterized by wide bandwidths and lower peak frequencies (Figs 2.3 & 2.4). Southern refugia cluster together across both Dimensions in multivariate space, but more so across Dimension 1 (Fig. 2.3). Northern refugia and the BMC are distinctly divergent from the southern refugia in both dimensions (Fig. 2.3 & 2.4). However, the northern refugia are also quite different from one another across both Dimensions, with the BMC falling in-between northern and southern refugia across Dimension 1 (Fig. 2.3 & 2.4).

MRPP analysis showed significantly larger variation among refugia than within refugia across multidimensional space, resulting in significant differences between refugia (MRPP test statistic $T = -118.506$, $n = 773$, $p < 0.0001$). In addition, variation among LR locations was significantly greater than within locations, indicating that Lamb Range locations are also significantly different in multidimensional space ($T = -39.622$, $n = 284$, $p < 0.0001$).

Table 2.4. Correlation (loading) matrix of all song variables used in the NMS analysis; *r*, *r²* and *tau* given for Dimension 1 and 2. *R²* of variables influencing song variation the most are indicated in bold.

Dimension	1			2		
	r	r²	tau	r	r²	tau
duration	-0.103	0.011	0.000	0.086	0.007	0.074
peak frequency	0.422	0.178	0.274	-0.918	**0.842**	-0.720
min frequency	-0.457	0.209	-0.287	-0.368	0.135	-0.275
bandwidth	0.891	**0.793**	0.676	0.426	0.182	0.360
notes/sec	-0.039	0.002	-0.050	-0.129	0.017	-0.094
# notes	-0.113	0.013	0.005	0.040	0.002	0.045
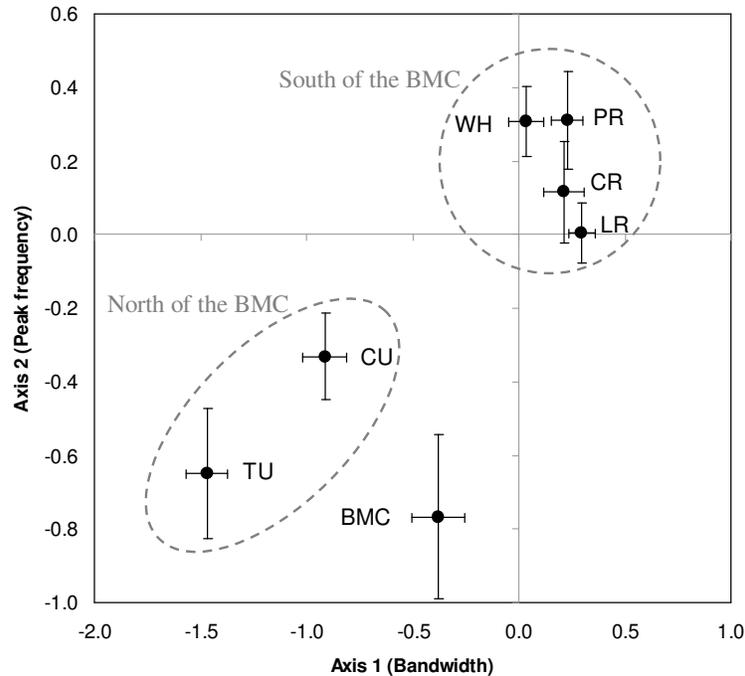
Figure 2.3. Ordination plot of the two significant NMS dimensions (Dimension 1 – Bandwidth; Dimension 2 – Peak frequency) using six spectrotemporal song variables of Chowchilla song; songs from each refuge and area of recolonisation* are represented by their centroid ± 2 S.E. (TU – Thornton Uplands, CU – Carbine Uplands, BMC* – Black Mountain Corridor, LR – Lamb Range, WH – Walter Hill Ranges, CR – Cardwell Range, PR* – Paluma Range).

Separate one-way ANOVAs confirmed that refugia were significantly different for Dimension 1 scores (ANOVA: $F_{6, 773} = 147.551$, $p < 0.001$) as well as for Dimension 2 scores (ANOVA: $F_{6, 773} = 30.241$, $p < 0.001$). Moving from north to south, bandwidth increased (Fig. 2.4a), and peak frequency decreased (Fig. 2.4b). For Dimension 1 (bandwidth) southern refugia (LR, WH, CR and PR) were not significantly different from one another with the exception of LR and WH, but southern refugia were all significantly different from the northern refugia (TU and CU) and the BMC (Fig. 2.4a). Similarly for Dimension 2 (peak frequency), the northern refugia and the BMC were again significantly different from the southern refugia, although the pattern was less clear. Three of the southern refugia (WH, CR and PR) were not significantly different from one another. However, LR was intermediate between the northern and these three southern refugia (Fig. 2.4b).
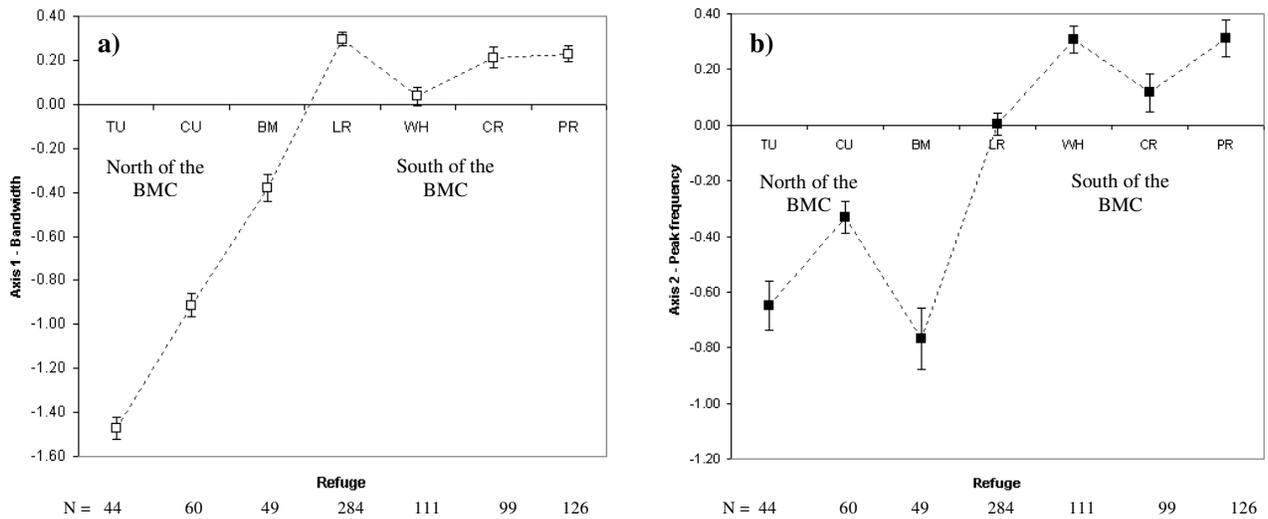
Figure 2.4. Ordination plot of the two significant NMS dimensions (Dimension 1 – Bandwidth; Dimension 2 – Peak frequency) using six song variables of Chowchilla song; songs from each refuge and area of recolonisation* are represented by their centroid ± 2 S.E. (TU – Thornton Uplands, CU – Carbine Uplands, BMC* - Black Mountain Corridor, LR – Lamb Range, WH – Walter Hill Ranges, CR – Cardwell Range, PR* – Paluma Range).

When plotting average NMS scores for each site/vegetation type in multidimensional space, there was no clear clustering of sites according to vegetation type (Fig. 2.5). Variation within vegetation types was as great as among vegetation types (Fig. 2.5).

Song dissimilarity was significantly correlated with geographic distance for between-refugia comparisons (Mantel test $r = 0.393$, $p = 0.011$), but not for within-refugia (LR) comparisons ($r = 0.213$, $p = 0.264$), indicating that song dissimilarity was influenced by isolation in refugia but not geographic distance within refugia (Fig. 2.6).

There was no significant correlation between body mass, body size or bill length and peak frequency or bandwidth (Fig. 2.7a & b; mass *vs.* peak frequency and bandwidth: $r^2 = 0.001$, $F = 0.001$, $p = 0.977$ and $r^2 = 0.015$, $F = 0.0891$, $p = 0.776$, respectively; tarsus *vs.* peak frequency and bandwidth: $r^2 = 0.272$, $F = 2.241$, $p = 0.185$ and $r^2 = 0.007$, $F = 0.046$, $p = 0.837$, respectively; *log* bill length *vs.* peak frequency and bandwidth: $r^2 = 0.023$, $F = 0.139$, $p = 0.723$, and $r^2 = 0.018$, $F = 0.111$, $p = 0.750$, respectively).

38
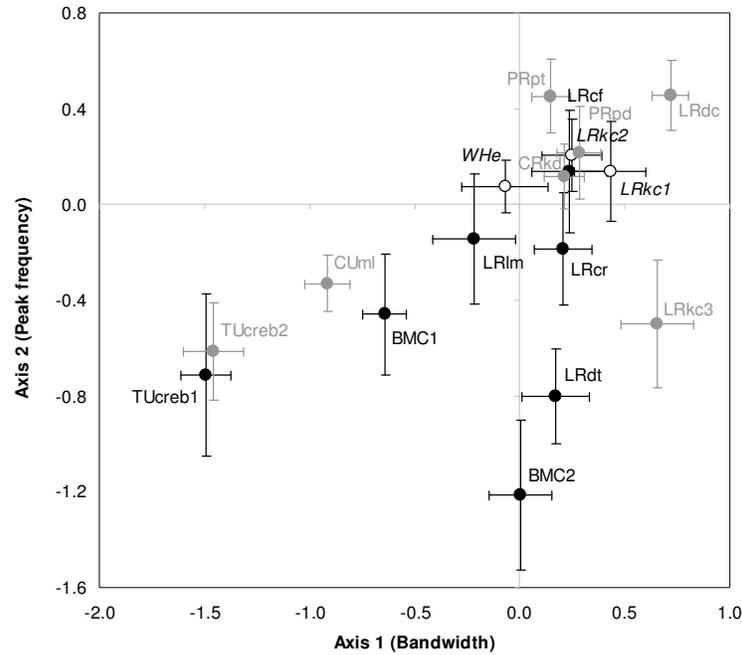
Figure 2.5. Ordination plot of the two significant NMS dimensions (Dimension 1 – Bandwidth; Dimension 2 – Peak frequency) of Chowchilla song; songs from each location are represented by their centroid ± 2 S.E. and identified by their respective vegetation type following Tracey's (1982) vegetation classification (Vegetation type 1 a & b – open circle; Type 2 a – filled circle; Type 8 – grey circle).
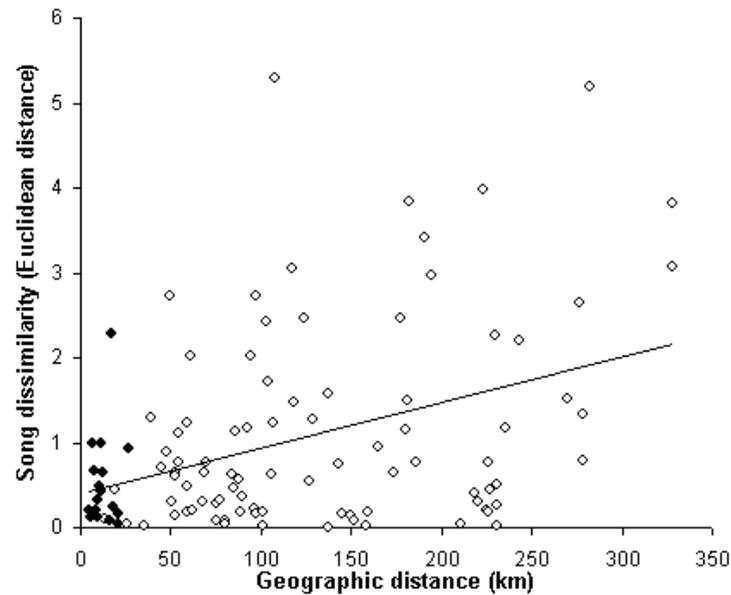
Figure 2.6. Pair-wise comparison of song dissimilarity (squared Euclidean distances) *vs.* geographic distance (km) for between/within refuge comparisons (within refuge comparison – closed circles, between refuge comparison – open circles); line of best fit for between-refuge comparisons (Mantel *r* = 0.393, *p* = 0.011).
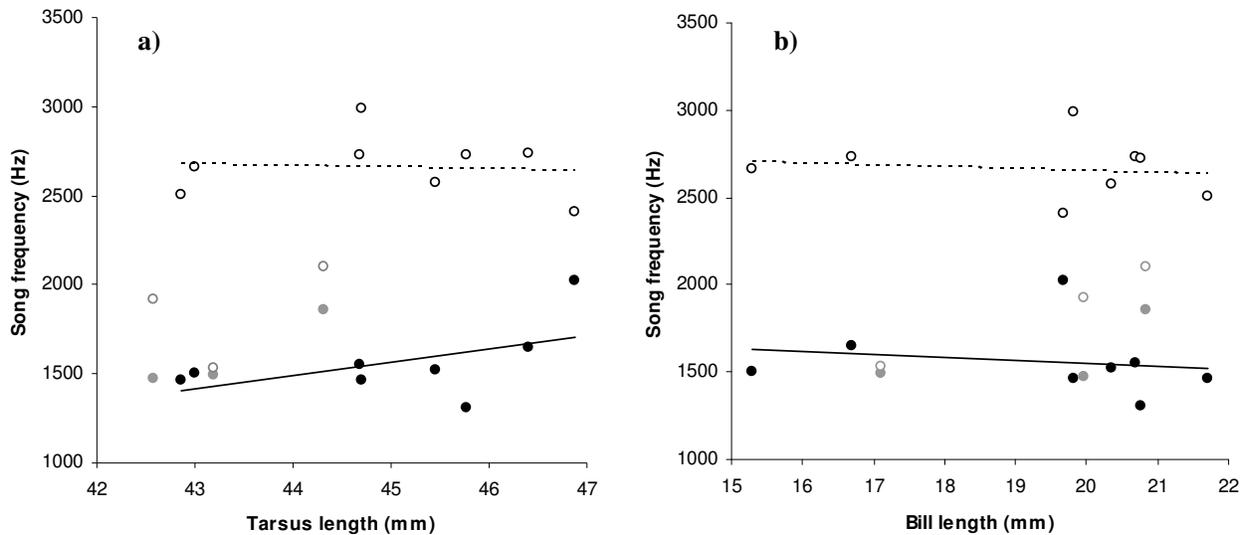
Figure 2.7. Linear regression of Chowchilla tarsus length (a) and bill length (b) *vs.* peak frequency (filled circles) and bandwidth (open circles); data for birds from north of the BMC are indicated in grey (peak frequency – filled circles, bandwidth – open circles); all relationships were non-significant (tarsus length *vs.* peak frequency and bandwidth: $r^2 = 0.272$, $F = 2.241$, $p = 0.185$, and $r^2 = 0.007$, $F = 0.046$, $p = 0.837$, respectively; *log* bill length *vs.* peak frequency and bandwidth: $r^2 = 0.023$, $F = 0.139$, $p = 0.723$, and $r^2 = 0.018$, $F = 0.111$, $p = 0.750$, respectively).

2.4   Discussion

This study clearly shows that Chowchilla song varies greatly across larger scales, and that refugial isolation indeed influenced spectral song characteristics in the Chowchilla.

Visible differences in Chowchilla song on spectrograms as well as easily audible differences include changes in the type, order and proportion of elements in the song, and also changes in the frequency range of the songs (Figs 2.2, 2.3 & 2.4). Songs from different refugia differed significantly in frequency characteristics, with a broadening of the bandwidth, as well as decreasing peak frequency when moving from north to south within the species' range (Fig. 2.4). songs from southern refugia cluster together closely in multivariate space (Figs 2.3 & 2.4), indicating that at this large scale, songs within the southern refugia are very similar, particularly in bandwidth. Spectral characteristics of songs from the northern refugia are distinctly set apart from the southern, although northern refugia are also significantly different from one another (Figs 2.3 & 2.4). Song from the BMC is more similar to the northern than the southern refugia, which may be explained by the fact that the recording sites were at the northern end of the BMC and could have been influenced by the northern refugia through recolonisation or simply proximity.

These results support the view that vicariant isolation can lead to a change in song characteristics. Isolation-by-distance does not seem to have had an effect on spectral characteristics. In addition, these results indicate that frequency characteristics in the Chowchilla may be influenced by similar processes to those driving genetic divergence. The distinct split in song characteristics across the BMC is indicative of the long isolation between the northern and southern populations. This divergence in song characteristics across the BMC mirrors the preliminary genetic and morphological divergence suggested previously in the Chowchilla (Joseph et al. 1995; Schodde and Mason 1999).

These findings raise the question about which forces drove the change in song frequency after populations were isolated. Spectral characteristics often change in different habitats, or due to the effect of body size or mass, none of which seemed to have influenced Chowchilla song frequencies (Figs 2.5 & 2.7). Previous studies on the influence of habitat differences on song were most often conducted across vastly different habitats (e.g. dense rainforest versus open ecotone forest; Slabbekoorn and Smith 2002b). In this study, all recording locations were within very similar rainforest habitats, and although these were of different types as classified by Tracey (1982), their classification is probably too broad a scale to draw definite conclusions. I found no effect of broad habitat type at the large scale of this study. At this larger scale, songs sung from within the same habitat type differed vastly between refuges (e.g. TUcreb & LRdc; Fig. 2.5), yet in some instances songs sung from different habitats within the same refuge were almost identical in their spectrotemporal characteristics (e.g. LRcf & LRkc; Fig. 2.5). Although I did not test for and thus cannot discount the possible effect of local-scale habitat differences on song characteristics, Kroon & Westcott (2006) did not find any evidence for local song dialects of the Golden bowerbird, *Prionodura newtoniana*, to be better adapted to the local acoustic environment. The Golden bowerbird shares its range and upland rainforest habitat with the Chowchilla, and both exhibit song characteristics superiorly adapted for long-range communication in dense forest (Richards and Wiley 1980; Endler 1992; Ryan and Kime 2002). Therefore, although I cannot dismiss the effects of small-scale habitat structure on local song variation, they seem unlikely to affect the large-scale song divergence found in this study.

The second alternative is that song characteristics changed due to morphological differences north and south of the BMC. Southern populations have larger body sizes than northern (see Chapter 4), and larger body size and body mass has been shown to inversely influence dominant or peak frequencies and bandwidth (Ryan and Brenowitz 1985; Wiley 1991; Badyaev and Leaf 1997; Doutrelant et al. 2001). However, within the southern refugia neither peak frequency nor bandwidth were correlated with body size or body mass in the Chowchilla (Fig. 2.7a). The slight but non-significant tendency of peak frequency to increase with body size (Fig. 2.7a) is opposite to the effect predicted. In addition, larger beaks have been shown to produce songs of lower minimum and maximum frequencies as well

as narrower bandwidths (Palacios and Tubaro 2000; Podos et al. 2004b; Huber and Podos 2006). Again, there was no relationship between peak frequency or bandwidth and beak length in the Chowchilla (Fig. 2.7 b). Therefore, the influence of Chowchilla morphology on song characteristics is unlikely, at least south of the BMC. Although scarce, data from north of the BMC does not show any of the predicted responses (Fig. 2.7a & b). When taken together, northern data fall well within the patterns shown for southern birds (Fig. 2a & b).

A different hypothesis is that of cultural drift in isolated populations, resulting in a random change of song characteristics over time. Such cultural drift can lead to the random fixation of a few song types or note types in the isolated population (Lynch and Baker 1994; Grant and Grant 1996; Podos et al. 2004a). Cultural drift seems the most likely explanation for the differences in song spectral characteristics between refugia, given the known history of population isolation that also resulted in genetic divergence in many other Wet Tropics endemics (Joseph et al. 1995; Schneider et al. 1998; Schneider and Moritz 1999). The results also show that song is more divergent between northern refugia than between southern refugia. TU is distinctly different from CU, especially in Dimension 1. In contrast, none of the southern refugia are clearly as divergent as the northern. This could be due to possibly longer isolation between northern refugia, or it may suggest higher levels of gene flow between southern refugia. Comparatively higher levels of gene flow south of the BMC are possible, given that most of the southern refugia were recolonised from the Lamb Range (Schneider *et al.* 1998). However, molecular genetic analyses are needed to confirm this hypothesis (see Chapter 4).

Using whole-song spectral characteristics may not give the necessary resolution to clearly determine the processes driving divergence at smaller scales, i.e. within refugia and between and within locations. The level of resolution of whole-song data is similar to that of previous genetic data, which was also unable to clarify any small-scale patterns of variation. Thus, further study is needed to resolve the forces driving song, genetic and morphological divergence in the Chowchilla in more detail and at a smaller scale.

In conclusion, this study clearly shows that spectral song characteristics were at least in part influenced by historical isolation in refugia, as historically isolated populations can be clearly distinguished by their spectral characteristics. Given the known history of population isolation in these refugia, as well as the clear pattern of very low song divergence at the Paluma Ranges despite similar or lower levels of connectivity to those of the BMC, cultural drift is the most likely explanation for the differences in spectral characteristics in Chowchilla song. The rainforests of the Wet Tropics are thought to be currently largely connected, and finding such clear divergence in Chowchilla song despite current population connectivity raises many interesting questions about the maintenance and consequences of such variation. For instance, do genetic and morphological patterns of divergence match the pattern of song divergence, and are these also mainly influenced by drift? Do Chowchillas

discriminate between different song variants? Exploring the similarity and order of song elements within Chowchilla songs may give a greater resolution of geographic patterns at the smaller scale. This will be the focus of the next Chapter.

CHAPTER 3   Small-scale Song Variation

3.1   Introduction

Inaccurate copying during the learning process can result in a gradual decline of song similarity with distance (Slater 1989). However, a multitude of other spatial patterns of song variation have also been found. This includes, for example, patchy song neighbourhoods or song dialects where groups of birds share the same or similar songs, which differ from the songs of other groups (Marler and Tamura 1962; Baker and Cunningham 1985). Many of these patterns cannot be explained by learning inaccuracies and distance alone. Proximate causes other than learning mistakes that are thought to influence the spatial pattern of song variation include the specific song learning strategy employed, the timing of song learning, migration and dispersal patterns, as well as the learning process itself (Goodfellow and Slater 1986; Slater 1989).

Dispersal and the timing of learning can strongly influence the pattern of geographic variation. For example, high levels of song sharing among immediate neighbours combined with a rapid decline in song sharing over short distances may occur when birds learn their song after dispersing and then match their song to that of their new neighbours, especially when birds learn from a single neighbour only and/or in the presence of geographic discontinuities (Goodfellow and Slater 1986; Slater 1989; Kroodsma et al. 1999; Wilson et al. 2000). Modelling has shown that song neighbourhoods comprising a relatively small number of birds separated by stable dialect boundaries can develop in such a way (Goodfellow and Slater 1986; Slater 1989). This pattern of song sharing has been found in Great tits, *Parus major*, (McGregor and Krebs 1982, 1989), Village indigobirds, *Vidua chalybeate*, (Payne 1985) and Corn buntings, *Miliaria calandra* (McGregor 1980; Latruffe *et al.* 2000).

On the other hand, when birds learn their song before dispersal, move short distances to find a vacant territory and preferentially try to settle near their tutors, there should be a gradual decline in song sharing with distance, with large variation in song sharing between neighbours at shorter distances, including neighbours sharing few or no songs or song types (Goodfellow and Slater 1986; Slater 1989; Wilson et al. 2000). Such a spatial pattern may also indicate a bird's quality, as more dominant birds may be able gain territories closer to the tutor. This pattern of gradual decline in sharing has been found in the Song sparrow, *Melospiza melodia* (Nordby *et al.* 1999).

These spatial patterns of song variation and their predicted learning and dispersal modes have been introduced by Slater (1989) and recently re-defined by Wilson *et al.* (2000). Wilson *et al.*'s (2000) model A (Fig. 3.1a) describes the first pattern of a steep, non-linear decline in song similarity across distance, indicative of post-dispersal and/or open-ended learning. Wilson *et al.*'s (2000) model B (Fig. 3.1b) describes the second pattern of a general linear decline with distance where close neighbours show moderate levels of sharing with high levels of variation, resulting in a triangular relationship. Wilson *et al.* (2000) also introduce a third model (C) of high sharing at intermediate distances but low levels of sharing at shorter and greater distances (Fig. 3.1c). This pattern is indicative of non-random dispersal or active avoidance of song sharing by neighbours. Although not mentioned by Wilson *et al.* (2000), some intrinsic assumptions in these spatial song patterns and their associated learning and dispersal strategies are stable territory sizes as well as stable distribution of dialect areas.

Although many studies have tried to clarify the causes of spatial variation in song, the relative importance of learning and dispersal strategies as well as other social influences are still unclear, particularly in cooperative passerines where females actively contribute to song contests and duets, e.g. Eastern whipbirds, *Psophodes olivaceus* (Mennill and Rogers 2005), Australian magpies, *Gymnorhina tibicen* (Brown and Farabaugh 1991), Black-bellied wrens, *Thryothorus fasciatoventris* (Logue et al. 2007), and White-browed sparrow weavers, *Plocepasser mahali* (Voigt et al. 2006). A detailed study of spatial patterns of song variation can give an understanding of the proximate reasons for such variation, which in turn may lead to an understanding of the ultimate evolutionary causes and/or the specific learning strategy employed. Few studies have focussed on the ultimate causes of such variation. Similarly, the evolutionary consequences of specific learning strategies and their associated patterns of song variation are also unclear, particularly, whether the occurrence of song dialects may in fact influence population genetic structure by reducing gene flow between sub-populations (Baker and Cunningham 1985; Slabbekoorn and Smith 2002a; Ellers and Slabbekoorn 2003).
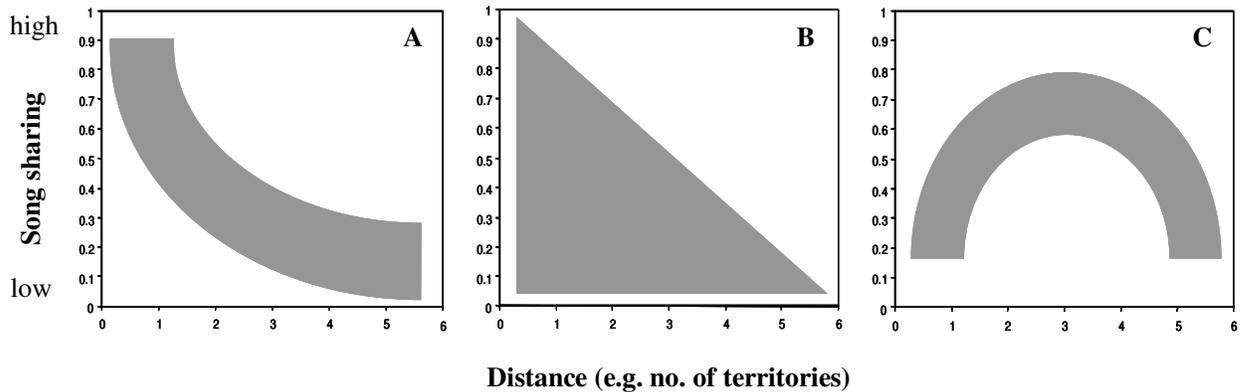
Figure 3.1. Three models of the relationship between song similarity and distance between individuals (modified from Wilson *et al.* 2000); this distance may be number of territories or geographic distance (e.g. meters); model A is indicative of post-dispersal and/or open ended learning, model B is indicative of pre-dispersal and/or closed-ended learning, and model C is indicative of non-random dispersal or active avoidance (Wilson *et al.* 2000).

The social and behavioural differences in singing behaviour of cooperative birds, as well as the lack of research into the song patterns of cooperative birds, provide a unique opportunity to study the applicability and subtleties of the proposed spatial patterns and learning strategies. In this study, spatial patterns of small-scale song similarity were established for the Chowchilla. These patterns were then compared to existing models to predict the timing of song learning and dispersal strategies in this tropical songbird.

Thus, the aims of this Chapter are to:

1) determine how and at what scale song similarity changes across geographic distance;

2) establish whether this pattern of change is indicative of the dispersal strategy and timing of song learning in the Chowchilla; and

3) infer the most likely evolutionary processes associated with such a strategy.

3.2 Methods

3.2.1 Study Sites

Details of the experimental design and location of recording sites can be found in Chapter 2 (Section 2.2). For this study, I focussed on six locations within the Lamb Range (LR: Douglas Track, dt; Clohesy Fig, cf; Clohesy Road, cr; Lake Morris, lm; Davies Creek, dc; and Kauri Creek, kc). Distances between these locations ranged from four to 27 kilometres. Four additional locations were situated in the Paluma Range (PR, 2 locations), Thornton Uplands (TU, 1 location) and in the Black

Mountain Corridor (BMC, 1 location). Full details on these locations can be found in Fig. 1.1 and Table 2.1.

3.2.2 Song Recordings

Song recordings followed the same methods as described in Chapter 2 (Section 2.2.2).

3.2.3 Syllable Classification

In order to analyse song structure, song similarity or sharing, and singing behaviour, it is essential to develop a song library for individuals, populations or species (Kroodsma and Miller 1982; Brunton and Li 2006). This library may, depending on the species and singing behaviour, consist of song elements, syllables or song types. Individual Chowchillas sing only one song type, and this song is made up of a number of distinct syllables (Fig. 3.2; see also Fig. 2.1). Here, I followed Mundinger's (1975) definition of syllables as the "basic structural units of a song, separated from adjacent syllables by a silent period of 0.02 to 0.2 seconds." Following this definition, syllables can be made up of one or several individual elements (i.e. single traces on a spectrogram). Chowchillas songs mainly consist of single-element syllables, although occasionally two-element syllables with a gap of less than 0.2 seconds can be found. Because of the prevalence of single-element syllables, I use the term 'element' more frequently than 'syllable' in this thesis. Nevertheless, if two elements were separated by less than 0.2 seconds, the two (or more) elements were classified and analysed as a whole unit.

Songs of each group of Chowchillas were divided into their elements/syllables and stored electronically as a spectrogram for each separate element, coded with the group's identity and location. I then visually sorted these spectrograms according to the similarity of element shape and frequency such that within- element type variation was lower than between element-type variation. Element types within each category were identified by individual numbers.

Although there are more objective methods of classifying song elements (e.g. Baker and Boylan 1995), visual classification was deemed an appropriate method of subdividing each song because Chowchilla songs have such visually distinct elements. However, to cross-check and validate this subjective classification, 19 spectrotemporal characteristics were measured for each element using the automatic measurement tool in Avisoft SAS Lab pro (Specht 2005). A Principal Components Analysis (PCA) was performed to reduce the large number of variables into several independent components summarising the directions of greatest variation. The principal components were then used to determine whether the previously defined element categories were statistically different. In addition, syllable similarity was also measured using the auto cross-correlation function in Avisoft SAS Lab pro (Specht 2005). A one-way ANOVA was used to determine whether the average correlation coefficient between two elements was significantly higher within element categories than between them.
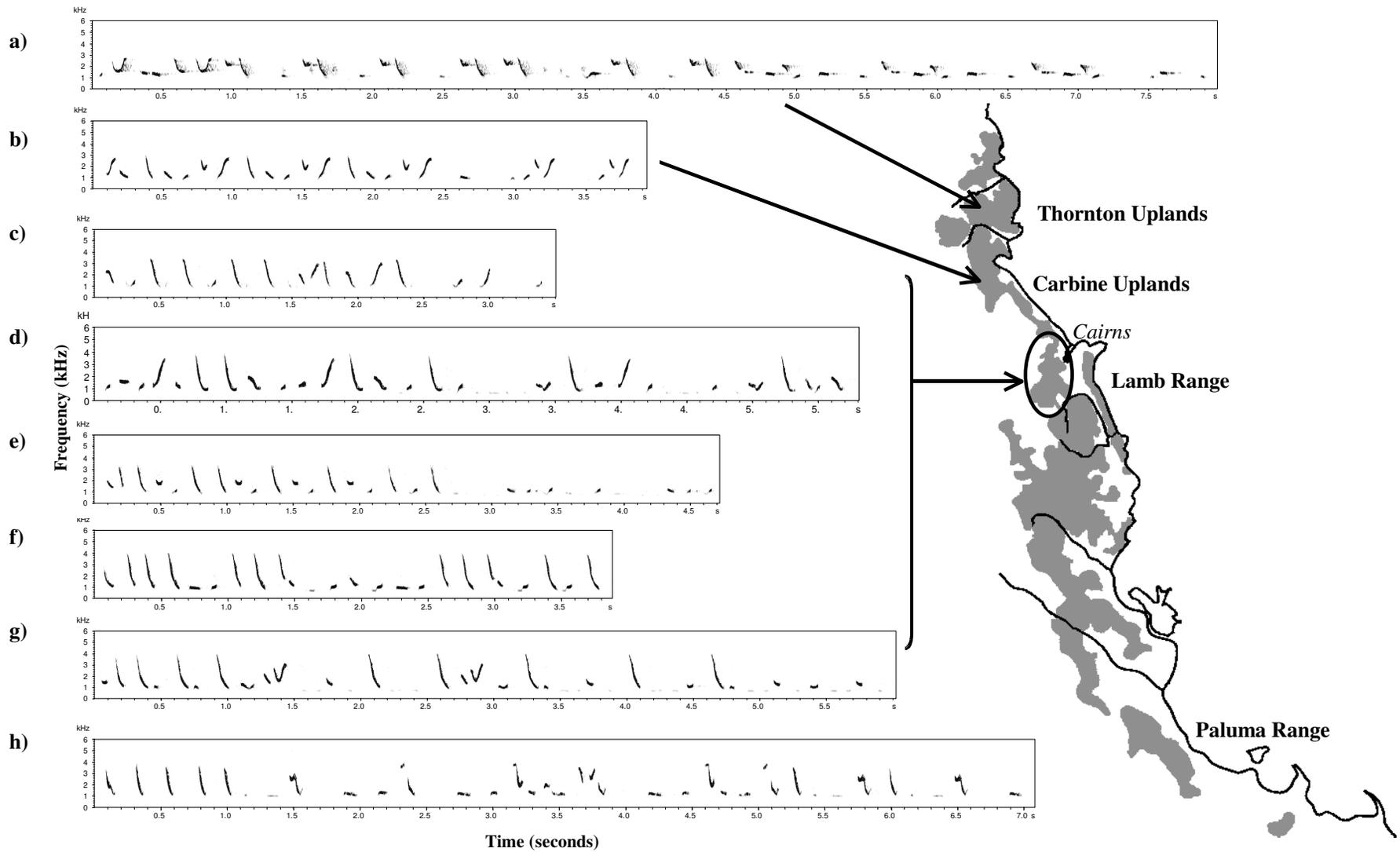
Figure 3.2. Examples of Chowchilla dialects from the Thornton Uplands (a), the Black Mountain (b), the Lamb Range (c-g) and Paluma Range (h). Spectrograms show songs from one of each site at a) Thornton Upland, b) Black Mountain, c) Douglas Track, d) Clohesy Fig, e) Clohesy River Road, f) Lake Morris, g) Davies Creek, g) Kauri Creek Road and h) Paluma dam.

3.2.4 Syllable Analysis

Each song was then transcribed into a string of identifying letters and numbers, and an element pool for each group was established. Chowchilla songs are composed of a fixed sequence of elements from this element pool. Each group's element pool was used to calculate an index of song element sharing between two groups of birds. This sharing index was calculated for each group of birds rather than for each individual bird because only individual groups could be confidently identified. Comparisons within groups (between birds) were not deemed necessary as all birds in a group always sing identical songs.

To determine song similarity between two groups, I calculated the standard song sharing index:

$$S = 2 N_s / (R_A + R_B),$$

where N_s is the number of elements shared in both groups' repertoires, R_A is the element repertoire of group A, and R_B is the element repertoire of group B (McGregor and Krebs 1982; Laiolo and Tella 2005). This sharing index is generally used to determine the number of song types shared between two birds, but has been applied to shared element types between groups here. The similarity or sharing index ranges from 0 (complete dissimilarity) to 1 (complete similarity). I calculated S for each group compared with every other group within each site, as well as with all other groups at all other sites. This resulted in a matrix of pair-wise comparisons between all groups ('groups x groups'). In order to reduce this 'group x group' matrix to a 'site x site' matrix I followed Tracy & Baker's (1999) method by averaging each group's S value over all groups at each site and subsequently averaging these means over all groups within each site. Thus, the mean S values progressed from group-group comparison, to group-site comparison, to site-site comparison. Group S values were used for within-site comparisons, whereas site S averages were used for between-site and between-refuge comparisons.

To test whether song similarity is correlated with distance, I used the non-parametric Mantel test, which evaluates the null hypothesis of no relationship between two similarity matrices (Manly 1997). The Mantel test is an alternative to regressing distance matrices that avoids the problem of partial dependence in the matrices (Manly 1997). The Mantel test statistic r was calculated using a Monte Carlo randomisation procedure with 5000 permutations. This test was performed using PC-Ord (McCune and Mefford 1999).

In addition, a regression analysis was performed for the relationship between *log* sharing index *vs. log* distance. The significance of each regression coefficient was tested using non-parametric resampling techniques (Resampling Stats, Inc. 2006). A frequency distribution of all possible values of the slope coefficient of each regression line was generated using 10 000 random associations between the

observed values of log sharing index and *log* distance. The position of the observed slope coefficient relative to the generated frequency distribution was then used to determine the confidence level at which this value could be considered significant.

In addition, the data was grouped into 'immediate neighbours' (neighbours in adjacent territories) and 'other neighbours' (neighbours from the same site but one or more territories removed), and 'non-neighbours' for both between-site comparisons and between location comparisons. Group *S* values were used for immediate and other neighbours, whereas site *S* averages were used for non-neighbours. The mean sharing index of each category was then compared with every other using the non-parametric Wilcoxon matched pairs and Tukey's HSD test. The statistical software JMP IN version 4.0.2 (SAS Institute Inc., Cary NC, U.S.A.) was used for these analyses.

Slater (1989) suggested that when analysing neighbour song sharing, one should compare immediate neighbours and those with one territory between them. Wilson *et al.* (2000) inferred the learning strategy of song sparrows from geographic patterns of sharing across up to seven territories between neighbours. Chowchilla territories are approximately 1-2 hectares for groups of three birds, with larger groups having larger territories (Jansen 1999). Hence, along a 1000m transect there should be approximately 5 to 10 resident groups. Therefore, this analysis only included within site and between site comparisons of up to 3 km, or approximately 15-20 Chowchilla territories.

Distances between sites were calculated using the distance calculator tool in a Garmin GPS unit (Garmin *etrex* Summit Version 2.06, Garmin Corp., Olathe, KS, U.S.A.).

3.3   Results

A total of 477 songs (LR: 258, PR: 126, BM: 49, TU: 44) from 53 groups (LR: 28, PR: 16, BM: 6, TU: 3) were transcribed into letter/number strings and used in the sharing analysis. The element pool of individual groups ranged from four to twelve elements with an average of 6.63 ± 0.57 elements per group (LR: 7.57, PR: 5.53, BM: 5.83, TU: 5 elements/group).

3.3.1 Syllable Classification

The classification resulted in an element catalogue of 56 different element types, which were divided into five broad categories (A, B, C, D and W; Fig. 3.3). The first four categories were sub-classified into element types. However, the fifth category of elements (W or 'warbles') was not sub-classified because of their comparatively low frequency and very low amplitude. This meant that recordings of these warbles were not of consistently good quality for confident sub-classification.
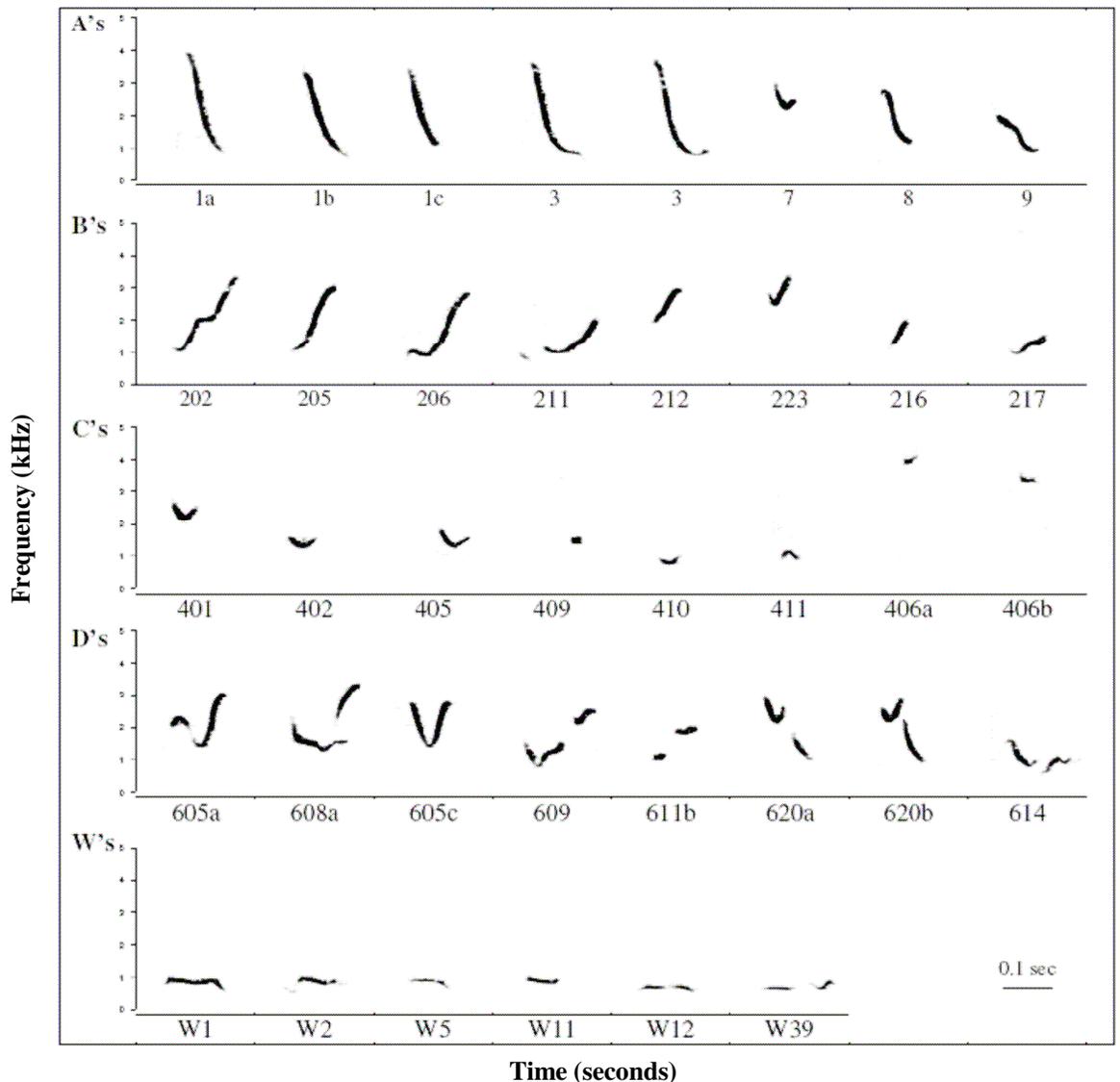
Figure 3.3. Chowchilla syllable examples, taken from the syllable/element library; syllables were classified into 5 broad categories, A, B, C, D and W; within those categories, syllables were grouped by similarity of frequency and shape. Numbers underneath syllables are syllable identifiers.

A-elements were defined by clearly descending frequency-modulation (high starting frequency, low end frequency) of varying bandwidth, ranging from very broad bandwidths (e.g. A1 notes, Fig. 3.3) to narrow bandwidths (e.g. A7, Fig. 3.3). B-elements were defined by clearly ascending frequency modulation (low starting frequency, high end frequency) that also varied in bandwidth (e.g. B202 and B216; Fig. 3.3). C-elements were those that either did not show any frequency modulation (e.g. C409) or those that had both descending and ascending parts in equal proportions resulting in u, v, or n shapes (e.g. C401 & C411; Fig. 3.3). W-elements were defined by very low frequencies (~1000-1200 Hz), very low amplitude compared to the other element types, and generally did not show any

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frequency modulation (e.g. W1, Fig. 3.3). D-elements were those that did not fit into the previous three categories and were generally unique to a location. D-elements were generally true syllables made up of one or more elements separated by less than 0.2 seconds (Fig. 3.3).

The PCA of 19 element characteristics resulted in four Principal Components with Eigenvalues greater than one, which together explained 86.8% of the variation (Table 3.1). The frequency at the start and end of an element explained most of the variation (61.6%, PC 1 & 2, Table 3.1). The bandwidth accounted for 12.7% of the variation (PC 3), and the peak amplitude at the start and end of the element explained 12.5% (PC 4). Using these Principal Components in a MANOVA showed that the five previously classified categories were distinctly and significantly different from one another (MANOVA: Willk's $\lambda_{16} = 0.227$, $F = 43.45$, $p < 0.0001$). In addition, the sub-categories within the four broad categories (A, B, C, D) were also significantly different from one another (MANOVA: Willk's $\lambda_{12} = 0.290$, $F = 43.52$, $p < 0.0001$).

The auto cross-correlation also supported my visual classification, as correlation coefficients were significantly lower between categories than within, for both the five broad categories as well as the sub-categories within those (between syllable categories: 0.183 ± 0.001; within syllable categories: 0.385 ± 0.004; between subcategories: 0.253 ± 0.022; within subcategories: 0.520 ± 0.180).

Table 3.1. Results of the Principal Components Analysis using 19 acoustic characteristics of each syllable in the syllable classification. Components with Eigenvalues >1 are shown, including the percentage and cumulative percentage, main influencing factors and general type of those factors. Components were rotated using Varimax Rotation

PC	Eigenvalue	%	Cum. %	Factors
1	6.374	33.55	33.55	Frequency at start of element
2	5.328	28.04	61.59	Frequency at end of element
3	2.408	12.68	74.26	Bandwidth of element
4	2.382	12.54	86.80	Peak amplitude at start & end

3.3.2 Syllable Sharing

There was a significant negative correlation between syllable sharing and geographic distance (Mantel: $r = -0.171$, $p = 0.0006$). Similarly, a linear regression of *log* sharing *vs. log* distance was also significant ($r^2 = 0.506$, $p < 0.0001$). Syllable sharing decreased sharply as distance increased (Fig. 3.4a). At a smaller scale, the sharp, non-linear decrease in levels of sharing over distance was more clearly apparent, with the largest drop occurring around one kilometre (Fig. 3.4b).

Immediate neighbours and other neighbours from the same neighbourhood (or site) had the highest level of syllable sharing (0.85 ± 0.09, $n = 21$, and 0.74 ± 0.07, $n = 58$, respectively), and they shared significantly more syllables than did non-neighbours. Non-neighbours from the nearest site shared significantly fewer syllables than neighbours but significantly more syllables than non-neighbours from further afield (Fig. 3.5; Wilcoxon: $\chi^2 = 110.40$, $p < 0.0001$; non-neighbours different site: 0.31 ± 0.03, $n = 57$; non-neighbours different location: 0.19 ± 0.03, $n = 36$). Again, the drop in the level of sharing occurred between neighbours within a site and non-neighbours between sites, which corresponds to a distance of approximately one kilometre.
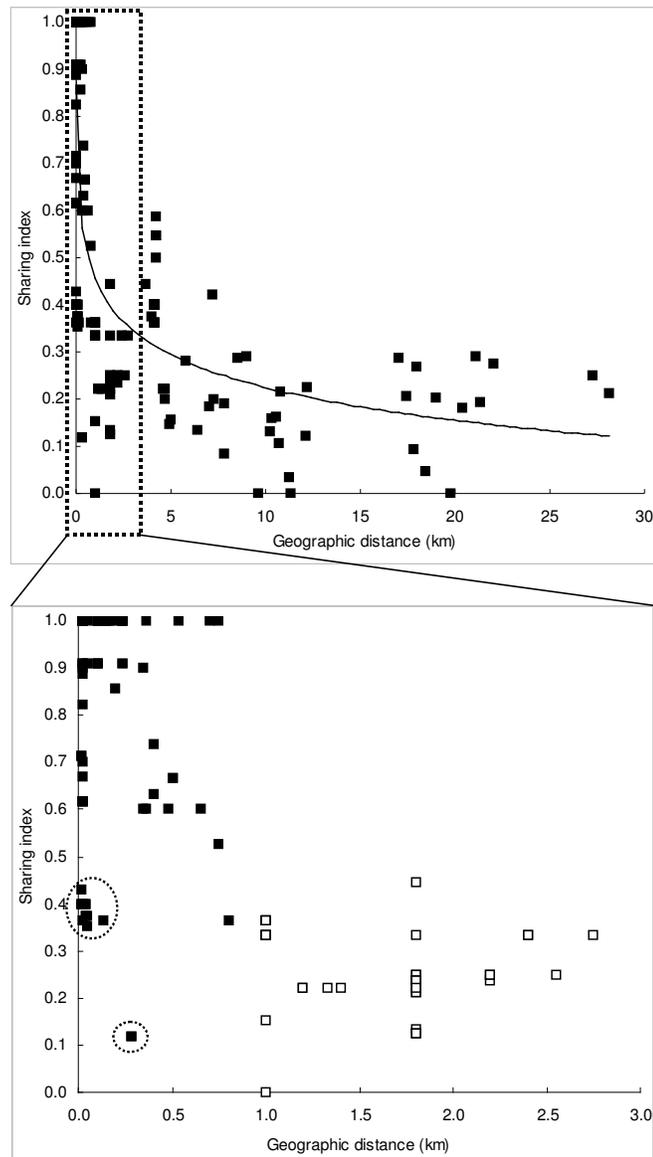
Figure 3.4. Song sharing index *vs.* geographic distance (km) for Chowchilla song; a) 0 – 30 kilometres; b) within site (= neighbours; closed squares) and between site (= non-neighbours; open squares) comparisons of up to 15 territories or 3 kilometres. Outliers are marked by dashed line (refer to Discussion for details).
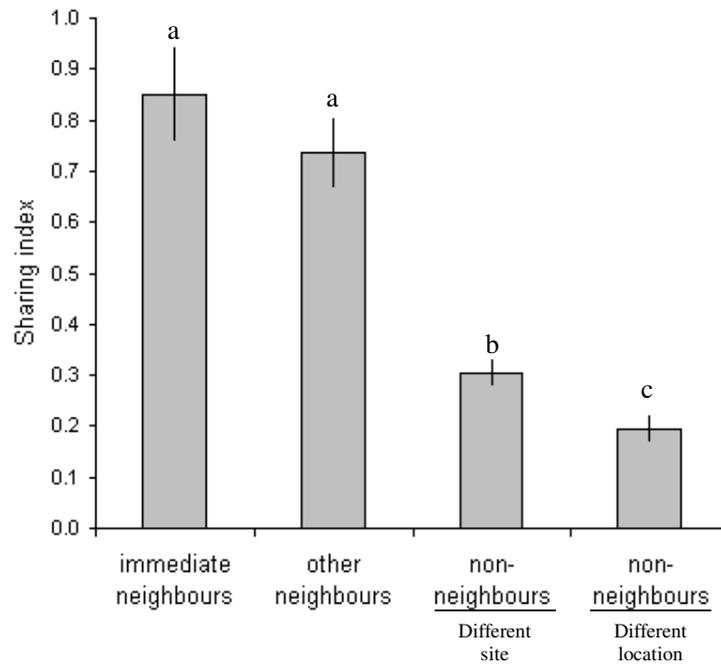
Figure 3.5. Song sharing index (± 2 SE) for immediate neighbours, other neighbours and non-neighbours (different site & different location) for within and between-site comparisons of Chowchilla song in the Lamb Range. Different letters indicate a significant difference, whereas the same letters indicate a non-significant difference at $\alpha = 0.05$.

3.4 Discussion

The results show that levels of song similarity in the Chowchilla were significantly higher among neighbours and decreased very rapidly and significantly with distance. The largest drop in syllable sharing occurred at a distance of approximately one kilometre between groups within a site (Figs 3.4 & 3.5). Sharing levels of non-neighbours from the next site were significantly different to those of non-neighbours from further away, indicating that a larger scale distance effect also occurred (Figs 3.4 & 3.5). This spatial pattern of song similarity in Chowchillas clearly falls into the category of a dialect mosaic, defined by low variation within dialects and large variation between with a clear boundary between dialects (Baker and Cunningham 1985). Levels of similarity decreased sharply at one kilometre, indicating clear boundaries between dialects. Therefore, Chowchillas form dialect neighbourhoods of several groups that sing mostly identical songs.

This microgeographic pattern of high levels of sharing amongst neighbours with a marked decline in sharing over short distances corresponds to Wilson *et al.*'s (2000) model A and suggests that Chowchillas acquire their song from their new neighbours after dispersing (Slater 1989; Wilson *et al.* 2000). However, the comparative analyses also show some variation between neighbours at closer distances and so do not clearly discount the possibility of an alternative learning strategy; that of

young birds dispersing only short distances while retaining their natal group's dialect. This corresponds to Wilson *et al.*'s (2000) model B. However, the number of occurrences of neighbours sharing few or no elements are very few, and the low levels of sharing at closer distances between a small number of groups may also be influenced by small-scale geographic discontinuities. If so, data points showing low sharing at close distances can be considered exceptions or outliers to the general pattern.

To more clearly distinguish between the two models I looked closely at groups that showed exceptionally low levels of sharing at relatively close distances. This occurred at two study sites, one within the Lamb Range and one within the Paluma Range. Within these sites, sharing levels between neighbours were almost as low as would be expected between non-neighbours. On closer inspection of the spatial arrangement of these groups, in all cases neighbours with low sharing levels were separated by geographic discontinuities such as frequently used roads and/or wider road clearings with a distinct gap in the canopy, and wider creeks (>10 m). In no cases did I find such low levels of sharing between immediate neighbours not separated by any geographic discontinuities. The importance of ecological barriers in driving dialect formation in songbirds has not been explicitly explored in the literature previously (Slabbekoorn, pers. com.). Therefore, although it is a clear pattern in the Chowchilla, I do not know whether this is a general pattern or one unique to the Chowchilla, and further research is needed to test this intriguing pattern.

Further support for the possibility that small-scale geographic discontinuities facilitate the maintenance of song neighbourhoods is the observation that groups of Chowchillas will approach but not cross either the wider roads or creeks during territorial encounters at these two sites (personal observation). Therefore, the sharing pattern in the Chowchilla seems more consistent with that of post-dispersal song learning, mainly due to the large and significant drop in sharing levels between neighbours and non-neighbours. This significant drop in similarity seems to occur where groups of Chowchillas do not directly compete with one another or confront one another in territorial contests.

Some studies have found a general, gradual decline in song similarity with distance previously (Beebee 2002). However, few studies have found a sharp decline in similarity over distance. Examples include the Great tit, *Parus major* (McGregor and Krebs 1982), Black-capped chickadee, *Poecile atricapillus* (Baker *et al.* 2000), Thrush nightingale, *Luscinia luscinia* (Griessmann and Naguib 2002), and the Green violet-ear, *Colibri thalassinus*, a non-passerine hummingbird (Gaunt *et al.* 1994). In these species, song and/or syllable sharing tended to be significantly greater among neighbours than between more distant birds or non-neighbours. However, the critical distance over which similarity declines differed with species. For instance, Great tits show a marked decline in similarity at distances of 100 to 200 metres, or one territory, whereas Black-capped chickadees show a similar but inconsistent decline across four kilometres (McGregor and Krebs 1982; Baker et al. 2000).

Few studies infer the song learning strategy and timing of learning from the spatial pattern of song sharing. Thrush nightingales are thought to form song neighbourhoods due to philopatry when returning to their old territories, rather than changing their song to match their neighbours (Griessmann and Naguib 2002). This suggests that Thrush nightingales learn their songs pre-dispersal and preferentially return to the area where they learnt their song (Griessmann and Naguib 2002). Song repertoires of the Great tit are composed of song types learned both pre- and post-dispersal. Those learned after dispersal are learned from their immediate neighbours (McGregor and Krebs 1982). Lastly, Green violet-ears are thought to modify their song to mimic their neighbours, suggesting possible post-dispersal learning (Gaunt *et al.* 1994).

Thus, this study is one of few that show such a clear, non-linear decline in song element sharing at a small spatial scale. The best explanation for this pattern currently available in the literature is that of post-dispersal song learning. However, I cannot discount the possibility that other, as yet unpublished mechanisms for this pattern may exist. Furthermore, Chowchillas differ from the other previously studied species showing this spatial song pattern, in that they only have one song in their repertoire and are sedentary, non-migrating birds. In addition, they live in cooperative groups where all group members of both sexes engage in territorial song year-round. This stands in stark contrast to most other species where single males defend territories during a relatively short breeding season.

Therefore, even though the spatial pattern of song in the Chowchilla seems to fit with the predictions of a post-dispersal learning strategy, it is important to be cautious about this conclusion. The models inherently assume that single male birds use their song to establish and defend their territory, as well as to attract a female, which does not sing. Although usually not mentioned, this assumption is inherent in many studies of bird song, especially those of high-latitude species. So it is surprising that such a model shows such a good fit for a cooperative, tropical species such as the Chowchilla, which has a distinctly different set of social factors affecting its behaviour. For instance, the traditional view of dispersing floaters trying to seize the territory of an established resident may not apply to cooperative birds that live in stable groups on stable territories for long periods of time. In addition, cooperative systems often show female-biased dispersal (Clarke et al. 1997; Cockburn 1998; Cockburn et al. 2003), which means that if song is learnt post-dispersal, it is the female that shows critical song learning abilities. Again, this stands in stark contrast to the traditional view of male-dominated song learning. Hence, the good fit of a model such as Wilson *et al.*'s (2000) for a cooperative bird is not only surprising and exciting, but it also shows that applying such models to different systems provides an opportunity to study the applicability and subtleties of previous theories of bird song.

This finding of distinct song neighbourhoods with an associated predicted learning and dispersal strategy in the Chowchilla leads to the question of the ultimate causes of such a strategy. Although it is not known how stable Chowchilla song neighbourhoods are over the long-term, it is known that Chowchillas occupy relatively stable territories for a long time (Jansen 1999) and that their dialects were stable over at least the four years of my study (personal observation). So high levels of sharing amongst neighbourhoods suggests that matching one's song to that of one's neighbours carries an advantage strong enough to enforce and maintain conformity across years.

There are three hypotheses that may explain high song sharing amongst neighbours that are compatible with my results. Firstly, the spatial pattern of Chowchilla song similarity may simply be a by-product of the song learning process itself. Chowchillas only have a single song type, live in small groups and tend to stay within stable territories, all factors that can facilitate dialect formation (Goodfellow and Slater 1986; Slater 1989). Modelling studies have shown that mosaic song neighbourhoods with sharp dialect boundaries are more likely to form when birds have only one or two song types, learn from a single neighbour only, learning accuracy is high and mortality low (Goodfellow and Slater 1986; Slater 1989). Goodfellow & Slater (1986) found that when modelling such a scenario, up to twenty territorial neighbours may share the same song type. The Chowchilla has only one song type in its repertoire, making it a prime candidate for dialect formation, independent of when they learn their song. Furthermore, because all birds within a group and all groups within a song neighbourhood sing identical songs, learning accuracy must be unusually high in this species, and birds probably learn selectively from their new group or the new group's song neighbourhood only. Although it may be possible that song similarity is a by-product of song learning, it is unlikely to be non-functional due to the aggressive nature of song-duelling between neighbours (see below). Nevertheless, this hypothesis can be tested using playback experiments to determine whether birds discriminate between different dialects (see Chapter 5).

Secondly, it has been suggested that song sharing with neighbours may be reproductively beneficial (McGregor et al. 1981; Payne et al. 1988; Beletsky and Orians 1989; Wilson et al. 2000). Payne *et al.* (1988) found that young Indigo buntings that shared their song with adult neighbours were more likely to mate than others of the same age. However, because Indigo buntings are open-ended learners it was unclear whether sharing levels between neighbours are high due to active copying of the neighbour or simply because they hear them more often (Payne *et al.* 1988). Similarly, this hypothesis was not supported in the Great tit (McGregor and Krebs 1984; McGregor and Krebs 1989). The evidence for reproductive success through song sharing is unclear and probably depends on the species studied. It is unknown whether song sharing in Chowchillas increases their reproductive success. However, it seems unlikely that song similarity directly influences breeding success due to the fact that all group members engage in song-duelling with neighbours, and such singing bouts would consume large amounts of energy that could otherwise be spent on reproduction.

The third and most likely possibility is that sharing songs may increase the effectiveness of territory establishment, maintenance and defence (Hill et al. 1999; Wilson and Vehrencamp 2001; Lachlan et al. 2004; Beecher and Brenowitz 2005). For example, neighbouring song sparrows respond more strongly to playback of shared song types than non-shared song types (Nielsen and Vehrencamp 1995), and territory tenure had been found to correlate with song sharing (Beecher *et al.* 2000). Thus, birds singing the same song type may be regarded as a more potent opponent, if immediate neighbours are more likely to pose a threat to one's territory than those further away. Hill *et al.* (1999) predicted that if song sharing is indeed important in territorial encounters, neighbours should share more songs than non-neighbours, and sharing levels should show a decline with distance. This prediction is directly supported by the findings in this study, and given the aggressive nature of Chowchilla territorial song-duelling, sharing is very likely to have an important function in the maintenance of Chowchilla territories.

This stands in direct contrast to the "dear-enemy-effect", according to which long-term neighbours are thought to respect mutual territory boundaries, saving time and energy by focussing defence efforts towards more threatening newcomers or floaters (Temeles 1994). The dear-enemy-effect assumes that a territory owner's potential losses are greater to newcomers than to neighbours (Temeles 1994). Therefore, it can be argued that because Chowchillas show higher sharing among neighbours, presumably for more effective defence against neighbours, potential losses to neighbours must be greater than those to newcomers. However, newcomers posing less of a threat than neighbours may seem counter-intuitive as newcomers would generally be expected to try to establish a breeding territory. Alternatively, since Chowchillas live in cooperative groups on very stable territories, newcomers may in fact be integrated into already established groups. If this is the case, birds dispersing from elsewhere, identified by their foreign dialect, may be perceived as potential helpers or mates rather than a threat, whereas established neighbours may be direct competitors for feeding or breeding resources. Jansen (1999) found that although core foraging areas of Chowchilla neighbours do not overlap, their home ranges do. Chowchillas also tend to shift their core foraging areas across seasons (Jansen 1999). This could lead to conflict with neighbours over loss of food resources within overlapping areas. Thus, if neighbours tend to pose a greater threat then high levels of song sharing and conformity amongst neighbours may indeed be related to more efficient territory defence. The low sharing at close distances for groups separated by identifiable, small-scale barriers also fits this theory. Chowchillas seem to be reluctant to cross barriers such as roads or creeks. In contrast, immediate neighbours may pose a threat to territory ownership; hence, extreme conformity would allow efficient territory defence.

Song sharing among neighbours may be explained by more effective territory defence, reproductive However, such song matching should in fact lead to reduced variation and matched songs across the

whole population (Lachlan *et al.* 2004). Therefore, while these models may identify the reasons for sharing among neighbours they cannot explain the occurrence of extreme dissimilarity between non-neighbours in Chowchillas. In line with the previously suggested model, one possible explanation for the continued maintenance of structural differences between non-neighbours is that dispersing birds may be perceived as potential helpers or mates and so preferentially incorporated into stable groups. If so, then the foreign dialect may identify a dispersing bird as such.

If this is indeed the case and song sharing is used to discriminate between and identify neighbours from strangers, Chowchillas would be expected to respond more strongly to local song variants than to increasingly distant and dissimilar song variants. This prediction will be tested using playback experiments in Chapter 5, in order to further clarify the functional significance of song variation in this species. However, before moving on to playback experiments, the next Chapter will explore the molecular genetic and morphological variation found in the Chowchilla and complete the analysis of large and small scale variation in this species.

CHAPTER 4   Morphological and Genetic Divergence

4.1   Introduction

The previous chapters aimed at determining the relative importance of different evolutionary forces in shaping large- and small-scale song divergence in the Chowchilla. In order to gain a more complete understanding of the evolutionary processes that influenced geographic divergence in this species, I will now examine the patterns of divergence in two additional character traits, morphology and molecular genetics. Taken together, the patterns of divergence in several character traits will reveal the evolutionary history of this species, and thus clarify the processes that initiate differentiation and divergence and, potentially, speciation.

In order to understand how populations diverge and ultimately form new species it is necessary to identify the relative contribution of different evolutionary processes to current patterns of genetic and morphological divergence. Global climatic fluctuations during the late Tertiary and Quaternary are thought to have been significant drivers of evolutionary change in many taxa and ecological systems through the processes of habitat contraction, fragmentation and subsequent re-expansion (Haffer 1969; Mayr and Ohara 1986; Moritz et al. 2000). Habitat contractions during glacial maxima caused populations to be isolated in refugia across climatic barriers for extended periods, while re-expansions following glacial retreat allowed subsequent founder events, range expansions and/or secondary contacts. These historical processes have been central in explaining current patterns of high biodiversity, endemism and divergence in tropical rainforest systems. In such systems, historical isolation in glacial refugia, on mountain tops or across large rivers are thought to have facilitated population divergence (Patton and Smith 1992; Fjeldsa and Lovett 1997; Moritz et al. 2000), but the relative importance of different evolutionary processes in ultimately generating and maintaining divergence and diversity is still highly debated. Furthermore, in contrast to the allopatric (refugial) hypothesis of divergence and speciation, there is also increasing evidence for the role of natural selection in promoting divergence and ultimately speciation in parapatric populations (Zink and Dittmann 1993; Smith et al. 1997; Schneider and Moritz 1999; Schneider et al. 1999; Smith et al. 2001; Smith et al. 2005).

As introduced in Chapter 1, Webb & Tracey (1981) proposed a number of small Pleistocene refugia throughout the Australian Wet Tropics (Fig. 1.1a). Alternatively, Nix and Switzer (1991) proposed

two larger isolates, which overlap many of Webb & Tracy's (1981) refugia (Fig. 1.1a). Both Nix and Switzer's (1991) isolates and Webb & Tracey's (1981) refugia are divided into northern and southern groupings by a much older, pre-Pleistocene climatic barrier known as the 'Black Mountain Corridor' (BMC; Fig. 1a; Webb and Tracey 1981; Nix and Switzer 1991; Hopkins et al. 1993).

Molecular genetic evidence suggests that this history of rainforest contraction and expansion (see Chapter 2) significantly affected the genetic structure of populations that were at one time restricted to these refugia. Many high-altitude, rainforest restricted endemics show a remarkably congruent pattern of strong mtDNA divergence across the BMC combined with patterns of expansion and (re)colonisation within the northern and southern isolates (Joseph et al. 1995; Winter 1997; Schneider et al. 1998; Schneider and Moritz 1999; Schneider et al. 1999; Hugall et al.).

However, despite clear evidence of long-term isolation resulting in molecular genetic divergence, there is a distinct lack of phenotypic differences among populations from north and south of the BMC (Schneider and Moritz 1999; Schneider et al. 1999; Nicholls and Austin 2005). This lack of phenotypic divergence has been shown in four endemic lizard species: *Carlia rubrigularis* (Schneider *et al.* 1998), *Gnypetoscincus queenslandiae* (Prickly skink), *Saltuarius cornutus* (Leaf-tail gecko), *Carphodactylus laevis* (Chameleon gecko) (Schneider and Moritz 1999), and more recently among a more volant and widely distributed bird species, the Satin bowerbird, *Ptilonorhynchus violaceus* (Nicholls and Austin 2005).

A consistent lack of phenotypic variation across the BMC has led to the conclusion that selective pressures within isolates must have been relatively stable, and that drift and founder effects were less important in creating phenotypic variation than previously thought (Schneider and Moritz 1999; Schneider et al. 1999). More generally, these findings have been incorporated into an increasing body of evidence that suggests that isolation through vicariance in rainforests is insufficient to produce phenotypic divergence among populations and that ecological differences are more important in promoting such divergence (Smith et al. 1997; Schneider et al. 1999; Smith et al. 2001; Smith et al. 2005).

To further investigate the effects of historical isolation on genetic and morphological divergence across the Wet Tropics rainforest, I examined patterns of both sequence variation and morphological variation in the Chowchilla. Two previous molecular studies on several Wet Tropics bird species, including the Chowchilla, showed a congruent pattern of divergence across the BMC. Net nucleotide divergence across the BMC in the Chowchilla was estimated to be 1.5% based on Restriction Fragment Length Polymorphisms (RFLPs; Joseph and Moritz 1994), and 2.4% based on *cytochrome b* sequences (Joseph *et al.* 1995). However, further conclusions on possible finer scale patterns of

divergence could not be made because of the small sample size and distribution in both previous studies.

The Chowchilla is also of special interest as phenotypic variation across the BMC has been suggested (Schodde and Mason 1999) but not statistically confirmed. Chowchillas are thought to be smaller north of the BMC with darker colouration of the head and back, and it has been suggested that two subspecies (*O. spaldingii melasmenus* north of the BMC and *O. spaldingii spaldingii* south of the BMC) exist based on these size and colour differences (Schodde and Mason 1999). In addition, comparing the patterns of genetic, morphological and song divergence will yield new insights into the influence of different evolutionary processes in this rainforest system.

Therefore, the aims of this Chapter are to:

1) investigate the patterns of morphological divergence in Chowchillas in order to determine whether this endemic species indeed shows statistically significant phenotypic divergence across the BMC;

2) determine the molecular genetic population structure and levels of divergence across the species' entire range; and

3) clarify the processes that may have caused morphological and genetic divergence and compare them with patterns of song divergence, thus re-evaluating the suggestion that isolation through vicariance in rainforests is insufficient to produce phenotypic divergence among populations.

4.2 Methods

4.2.1 Morphological analyses

Fifty-three Chowchillas were captured (25 females, 28 males) in five refugia and three areas of recolonisation across the species range (TU, $n = 2$; CU, $n = 9$; LR, $n = 17$; AU, $n = 2$; WH, $n = 6$; CR, $n = 2$; BMC, $n = 3$; PR, $n = 12$; see Fig. 1.1 and Chapter 1 for abbreviations). Chowchillas were captured using one to three large-gauge (63 mm) mist nets set up in a straight line or in the shape of a V or U depending on the terrain and vegetation density. Nets were set up so that the bottom edge of the net was flush at ground level. A combination of playback of local territorial song and Chowchilla alarm calls, as well as one person herding a group towards the net, yielded the highest capture rates. Once a Chowchilla was in the net, the playback was ceased, and the bird(s) immediately extracted from the net and placed in separate cotton bags. Morphological measurements as well as blood and feather samples were then taken *in situ*. Handling time was kept to a minimum and birds were released immediately after all measurements were taken.

Morphological measurements included body mass, length of tibiotarsus, length of unflattened wing chord, tail length, head-bill length and bill length (exposed culmen). Digital Vernier callipers (±0.05 mm) were used for all size measurements except wing chord length, which was measured using a ruler

(±0.5 mm). A Pesola$^©$ spring balance (±0.05 g) was used for mass measurements. Birds were sexed by their plumage. All captured birds were adults.

The normality of each measurement was assessed using Shapiro-Wilk's test. Data were *log*-transformed prior to analysis if deviations from normality were detected. Morphological measurements tended to be correlated with each other so I used a Principal Components Analysis (PCA) to extract a single composite variable of body size (the first Principal Component, PC1). This first Principal Component was then used in a two-way ANOVA with sex and location as factors. Sex was included as a factor in all analyses to test for significant sexual size dimorphism (SSD) among sampling locations.

In addition, I tested for morphological divergence between sexes and across the BMC for each morphological variable separately using two-way ANOVA as described above. Due to significant divergence in body size among locations (see Results, Fig. 4.1) the analyses were repeated on morphometric measurements scaled for body size (variable divided by tarsus size) to gain an understanding of morphological variation independent of allometric body size effects. Finally, Tukey's HSD post-hoc tests were used to determine whether birds from the BMC were more similar in morphological characters to northern or southern birds.

4.2.2 Molecular analyses

Samples were obtained from blood collected from most captured birds ($N = 51$) and from liver tissue samples stored at the Australian National Wildlife Collection at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Canberra ($N = 10$; FU, $n = 4$; TU, $n = 1$; CU, $n = 1$; AU, $n = 4$), covering six refugia and three areas of recolonisation in total. Whole blood was collected from the brachial vein of each captured Chowchilla and stored in Queen's lysis buffer (Seutin *et al.* 1991). Liver tissue from the museum specimens was frozen before sub-samples were taken and stored in ethanol.

Total cellular DNA was extracted from blood and liver tissue using a QIAGEN extraction kit and following the manufacturers protocol, with the exception that the recommended amount of Proteinase K was increased to 30 microliters (μL) per 20 μL of blood, and the digestion time was increased to 4 hours. For liver tissue extraction, digestion time was also increased to 4 hours. The quality of DNA was checked by loading 8 μL of the resuspended DNA product and 2 μL of loading dye (Bromophenol blue) into a 1% agarose gel for electrophoresis. Gels contained 0.004 μL of Ethidium bromide per ml of agarose to visualise the DNA under ultraviolet light (GelDoc 1000 image system, BIORAD). Gels were run in 1x TBE buffer at 100V for 20 mins.

Mitochondrial DNA haplotypes were identified by amplifying a 447 bp fragment of the hypervariable (Part I) control region by polymerase chain reaction (PCR), using primers CC16674L (5'-CATAGCCCAAGAAATAAGAC-3') and CC447H (5'-GGAGTCCGACTAATAAATAACC-3'). No control region primers were previously available for Chowchillas. Therefore, I developed species-specific primers by using generic avian primers and 'primer-walking', starting in the *cytochrome b* region of the Chowchilla, for which sequence data was available. PCR amplifications were performed in 25μL reactions containing 10x reaction buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 10 pmol of either primer, 0.1 mM of each dATP, dTTP, dCTP and dGTP, 0.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (*Life Technologies*) and 17.15 μL of purified water (ddH$_2$O). No less than 10 ng of DNA extract was used in each PCR reaction. Amplifications were performed using the following cycling conditions: initial denaturing step of 94ºC for 90s, followed by 35 cycles of denaturing at 94ºC for 30s, annealing at 55ºC for 60s, extension at 72ºC for 120s, and a final extension step at 72ºC for 7 minutes. The quality and size of the PCR product was determined by gel electrophoresis as described above, using 5 μL of PCR product and 2 μL of loading dye. The quantity of PCR product obtained was estimated using a precision molecular mass ladder (Bio-Rad) run on each gel according to the manufacturer's protocol.

PCR products were cleaned using spin column purification (UltraClean^{TM}, MO BIO Inc.) and subsequently sequenced *ex situ* at the Australian Genome Research Facility (AGRF) using BigDye^{TM} primer incorporation (Applied Biosystems). Sequencing products were purified and screened using an AB3730xl automated DNA sequencer.

4.2.3 Phylogenetic analyses

Sequences were aligned using ProSeq (Version 2.91, Filatov 2002). The significance of different hierarchical population genetic structures was tested using an Analysis of Molecular Variance (AMOVA) in ARLEQUIN (Version 3.1, Excoffier *et al.* 2005). Due to small samples sizes per location, samples were pooled into four populations that corresponded broadly to proposed Pleistocene refugia (Northern Uplands, FU, TU & CU, $n = 16$; Atherton Uplands, LR & AU, $n = 23$; Cardwell Ranges, CR & WH, $n = 8$; and Paluma Ranges, PR, $n = 12$). Using pair-wise F_{ST} comparisons, different hierarchical population structures were tested. In addition, I also tested for any apparent population structure without any *a-priori* hierarchical groupings, as well as for any sub-structuring between southern populations only. The most parsimonious population structure was considered to be that which maximised between group variance while simultaneously minimising variation between populations within groups (e.g. Congdon *et al.* 2000).

I tested the assumption of stable effective population size over time (inherent in methods estimating population genetic structure, including AMOVA) by conducting several neutrality tests. These

included mismatch distributions, Fu's *Fs* test, Fu and Li's *F** and *D** tests as well as Tajima's *D* test, which detect possible historical population growth, decline or stability.

Mismatch distributions determine the number and distribution of pairwise differences between all possible pairs of sequences or haplotypes in a sample, which gives an indication of population stability or growth (Slatkin and Hudson 1991; Rogers and Harpending 1992; Harpending et al. 1993). A unimodal mismatch distribution indicates rapid population expansion, whereas a geometric distribution indicates stable population sizes (Slatkin and Hudson 1991; Rogers and Harpending 1992; Harpending et al. 1993). A mismatch analysis was conducted for the main genetic groups as determined in the AMOVA. The mismatch distributions were compared with the fit to a Poisson model in ARLEQUIN 3.1 (Excoffier *et al.* 2005).

Fu's (1997) F_S explicitly tests for population growth by detecting an excess of the number of rare alleles compared to the number expected in a static population (Fu 1997). In addition, Fu's & Li's (1993) *F** and *D** tests distinguish between the effects of population growth and those of background selection (Fu and Li 1993; Fu 1997). Thus, patterns of population growth or selection can be distinguished by the significance of F_S, *F** and *D**: population growth is indicated when F_S is significant but *F** and *D** are not, whereas selection is indicated by the reverse trend. Lastly, Tajima's (1989) *D* analyses population sequence polymorphisms and can detect population expansions. This is based on the observation that under neutrality the number of nucleotide differences should be equal to the number of differences between segregating (polymorphic) sites (Tajima 1989; Aris-Brosou and Excoffier 1996). Thus, significant negative departures of Tajima's *D* from zero indicate population expansion.

Phylogenetic patterns of sequence divergence were also examined by pairwise distance analysis (Median Joining, MJ; Bandelt et al. 1999). A nearest-neighbour, median-joining network of haplotypes was constructed using NETWORK (Version 4.2; www.fluxus-engineering.com).

4.2.4 Divergence times

Population expansions were dated using the mismatch distributions and a non-linear least square approach in ARLEQUIN 3.1 (Rogers and Harpending 1992). The expansion time in mutational units (τ) was converted into years before present (bp) using the following formula:

$$T = \tau / 2\mu k * \text{generation time,}$$

where *T* is the expansion time (in years bp), μ is the mutation rate per site per year and k is the sequence length. There is considerable debate and uncertainty about divergence rates. The conventional divergence rate for coding regions of the mtDNA is 2% per million years (Shields and

Wilson 1987; reviewed in Klicka and Zink 1997). However, mutational rates of the non-coding control region have been found to be up to an order of magnitude higher, from 2% in Fox sparrows, *Passerella iliaca* (Zink and Weckstein 2003), 15% in Dunlins, *Calidris alpina* (Wenink et al. 1994), to 20% in Adélie penguins, *Pygoscelis adeliae* (Lambert et al. 2002) and 20.8% in the Canada goose, *Chen caerulescens caerulescens* (Quinn 1992). Similarly, Avise & Walker (1998) used a rate of 20% for a range of bird species. Thus, I estimated expansion times for a range of mutation rates (0.02, 0.15 and 0.20 substitutions/site/million years). Generation times of Chowchillas are also unclear, although they are likely to be relatively long for two reasons. Firstly, during this study I recaptured a female that was banded as an adult in 1990, which suggests that Chowchillas can live to at least 14 years of age. Secondly, Chowchillas live in social groups that cooperatively defend stable territories. Such group living and territoriality has been linked with high annual survival and longevity (Ridley *et al.* 2005). In addition, in such systems offspring often show delayed dispersal and may not gain a breeding opportunity until inheriting a territory (Kokko and Lundberg 2001; Ekman 2006; Griesser et al. 2006). Therefore, a range of generation times was used (1, 2 and 4 years) to estimate a range of possible divergence times.

4.3   Results

4.3.1 Spatial patterns of morphological variation

Measurements from 50 birds (23 females, 27 males) were used in the north/south morphological comparisons. Three birds from within the BMC were excluded from these comparisons, as they were neither from north nor south of the BMC. However, data from the BMC birds were taken into account and compared to the northern and southern birds separately.

Chowchillas showed significant sexual-size dimorphism (SSD) in all characters except for absolute bill length. Males were significantly larger and heavier than females, irrespective of the location (no interaction between sex and location; Table 4.1). In addition, there was significant SSD in relative bill and head sizes with females having larger bills and larger heads relative to their body size irrespective of the location (north *vs.* south of the BMC; Table 4.1). Because of this strong sexual dimorphism, all ANOVAs were performed using both location and sex as a factor.

Table 4.1. Patterns of morphological variation in Chowchillas north and south of the Black Mountain Corridor (N/S) and between the sexes; PC1 is a composite body size measure derived from a Principal Components Analysis. *F* and *p* are given for the two factors (N/S and Sex) as well as for the interaction effect.

	N/S		Sex		Interaction	
	F	*p*	*F*	*p*	*F*	*p*
PC1	54.008	**<0.001**	244.723	**<0.001**	1.853	0.181
Tarsus	8.938	**<0.001**	110.142	**<0.001**	2.363	0.131
Weight*	75.131	**<0.001**	170.735	**<0.001**	2.172	0.147
Bill*	0.229	0.634	0.498	0.484	0.086	0.770
Head*	1.238	0.272	9.959	**0.003**	0.180	0.673
Tail	12.706	**<0.001**	85.421	**<0.001**	4.323	**0.043**
Wing	0.481	0.491	46.084	**<0.001**	0.244	0.624
Rel. Weight*a	29.010	**<0.001**	34.727	**<0.001**	5.958	**0.019**
Rel. Bill	0.783	0.381	7.665	**0.008**	0.081	0.777
Rel. Head*b	1.622	0.210	6.973	**0.011**	1.843	0.182
Rel. Tail	0.001	0.973	0.722	0.400	0.338	0.564
Rel. Wing	8.431	**0.006**	7.547	**0.009**	3.331	0.074

log-transformed data; a *log*(weight/tarsus) & b *log*(head/tarsus)

When deriving a single composite variable of body size using a PCA, the first principal component explained 63.5% of the variation in the six morphological measurements and was influenced most strongly by weight and wing chord (component scores of 0.939 and 0.935, respectively). PC1 reflected body size well for both sexes, being strongly correlated with all variables except bill size for males, and bill and head size for females. Both sex and location significantly influenced PC1, indicating significant morphological variation across the BMC and between the sexes within each region (Table 4.1, Fig. 4.1a). Birds north of the BMC (northern birds) are significantly smaller than those south of the BMC (southern birds), and females are significantly smaller than males, irrespective of location (no interaction between sex and N/S; Table 4.1, Fig. 4.1a).

When analysing each morphological variable separately, absolute tarsus size, weight and tail length vary significantly with both sex and location (Table 4.1), supporting the PCA results. Northern birds are significantly smaller, lighter and have smaller tails than southern birds. Absolute bill, head and wing length do not vary across the BMC. When scaled for body size, relative weight and wing length also vary significantly with both sex and location. Northern birds are significantly lighter relative to their body size than southern birds. Notable, however, is the lack of variation in absolute bill size both between the sexes as well as across the BMC (Table 4.1, Fig. 4.1b).

Due to the small sample size obtained from within the BMC (*n* = 3; 1 male, 2 females), only female morphological variables were compared to northern and southern populations. Females from the BMC

were more similar to southern birds and significantly different to northern birds in tarsus length (Tukey HSD, $p = 0.034$). None of the other morphological variables measured were distinguishable from either the northern or southern population.
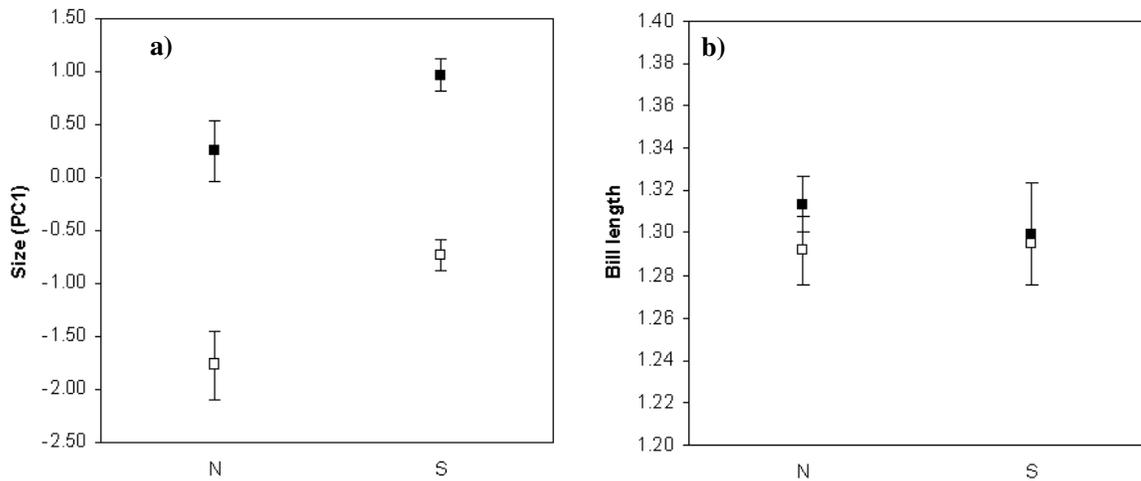
Figure 4.1. Morphological variation in male (■) and female (□) Chowchillas north (N) and south (S) of the Black Mountain Corridor; a) mean composite body score (PC1 score) ± 2SE; b) mean bill length ± 2SE.

4.3.2 Spatial patterns of genetic variation

The 62 Chowchilla mtDNA control region sequences showed 14 (0.035%) variable sites and 10 (0.025%) phylogenetically informative sites. There were no insertions or deletions, and all but one base changes were transitions. The one transversion observed was a singleton. I found 14 different haplotypes among the sequences (Table 4.2). These haplotypes fell into two distinct lineages separated by the BMC, which did not share any haplotypes (Table 4.2, Fig. 4.2). Patterns of mtDNA sequence divergence showed a distinct genetic break across the BMC (1.5% observed sequence difference).

Table 4.2. Variable nucleotide sites for the 14 haplotypes found in mtDNA control region sequences of 62 Chowchillas; number of individuals found with each haplotype are given for each geographical area. N – Northern Uplands (TU, CU & BT), AU – Atherton Uplands (LR & AU), CR – Cardwell Ranges (CR & WH), and PR – Paluma Ranges.

Haplotype	n				Nucleotide site													
	N	AU	CR	PR	99	120	183	242	260	285	286	280	301	306	303	326	320	330
h1	4				C	C	C	T	A	A	T	A	G	T	T	C	G	C
h2	5				.	.	.	.	.	.	.	.	A	.	.	.	.	.
h3	1				.	.	.	.	.	.	.	.	A	.	C	.	.	.
h4	2				.	.	.	.	.	.	.	.	A	.	.	.	A	.
h5	3				.	.	.	.	.	.	C	.	.	.	.	.	A	.
h6	1				.	.	.	.	.	.	.	.	A	C	.	.	A	.
h7		19	6	11	.	T	T	C	.	.	.	.	A	.	.	T	A	T
h8		1			A	T	T	C	.	.	.	.	A	.	.	T	A	T
h9		1	1		.	T	T	C	.	.	.	G	A	.	.	T	A	T
h10		1			.	T	T	C	G	.	.	.	A	.	.	T	C	T
h11			1	1	.	T	T	C	G	.	.	.	A	.	.	.	A	T
h12		1			.	T	T	C	.	G	.	.	A	.	.	T	A	T
h13		1		1	.	T	T	C	G	.	.	.	A	.	.	T	A	T
h14		1			.	T	T	C	G	.	.	.	A	.	.	T	A	T
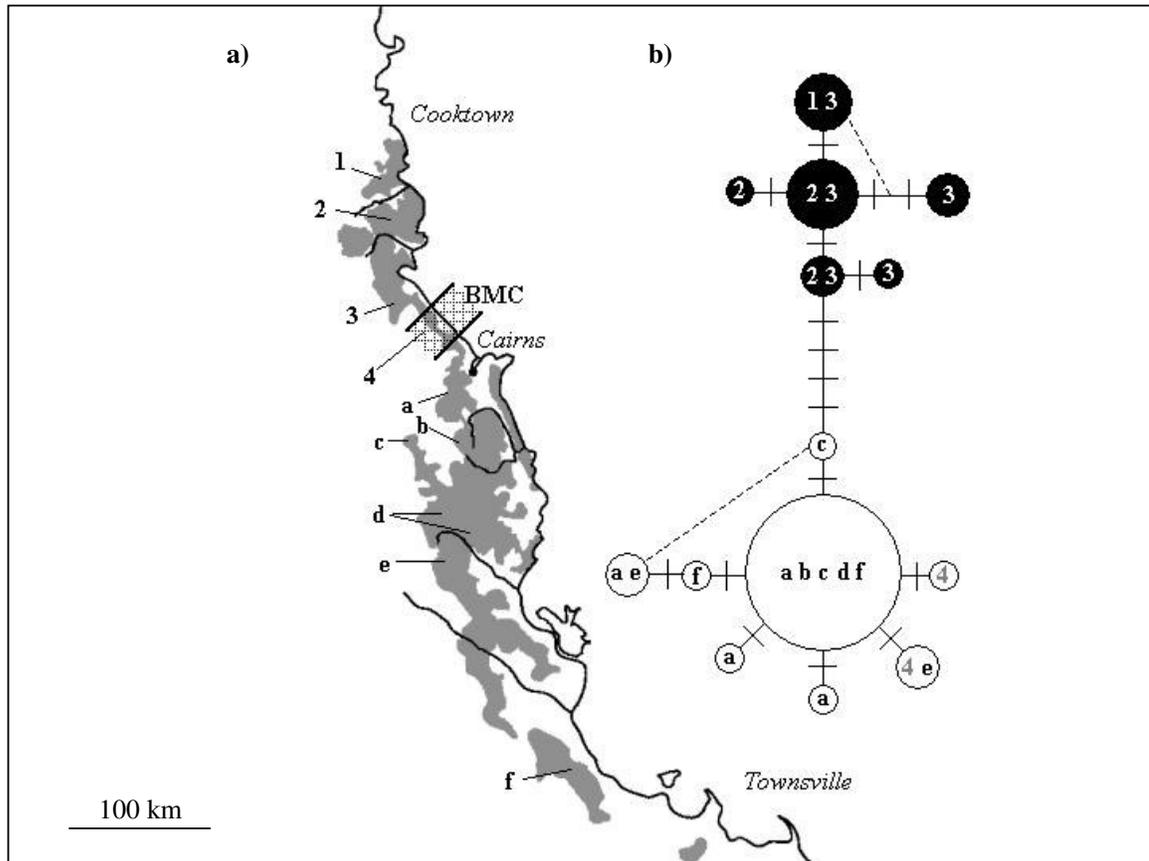
Figure 4.2. a) Map of the Wet Tropics World Heritage Area ('Wet Tropics') showing the current distribution of rainforest habitat (light grey) and sampling sites (northern refugia 1-3, BMC 4, southern refugia a-f). b) Parsimony network for mtDNA control region sequences of Chowchillas generated by nearest-neighbour joining; circle size denotes relative haplotype frequencies, circle colour denotes location north (black) or south (white) of the BMC, and dashed lines indicate potential homoplasies. 1 – FU, 2 – TU, 3 – CU, 4 – BMC, a – LR, b – AU, c – Mt. Baldy, AU, d – WH, e – CR, f – PR (refer to text for abbreviations).

Pair-wise analysis of F_{ST} values between all four populations showed very high F_{ST} values between the northern and the southern populations (0.799 – 0.855) but very low F_{ST} values between the southern populations (Table 4.3), revealing significant genetic subdivision among the northern and the three southern populations (AMOVA F_{ST} =0.783, df = 3, $p < 0.0001$). This non-hierarchical structure explained 78.3% of variation between and 21.7% of variation within populations. When sub-structuring populations into one northern and one southern grouping as suggested by the pair-wise analysis, this N/S structure was more parsimonious than the non-hierarchical structure, explaining 87.3% of variation between and 12.7% of variation within populations. This hierarchical structure revealed significant N/S genetic subdivision (AMOVA F_{ST} =0.873, df = 1, $p < 0.0001$). Other hierarchical structures were also tested but yielded less parsimonious results.

Table 4.3. Pair-wise F_{ST} comparison matrix for four Chowchilla populations: North, Atherton Uplands (AU), Cardwell Ranges (CR) and Paluma Ranges (PR). Significant comparisons (AMOVA: p <0.0001) are indicated by an asterisk.

	North	AU	CR	PR
North	0			
AU	0.855*	0		
CR	0.799*	-0.037	0	
PR	0.834*	-0.033	-0.072	0

Sequence divergence either side of the BMC was very low (Table 4.4). A widespread, shared ancestral haplotype, as well as a star-shaped parsimony network are expected under rapid and recent population expansion (Slatkin and Hudson 1991; Templeton et al. 1995). The southern populations clearly showed this star-like pattern consistent with rapid population expansion (Fig. 4.2). Fu's F_S as well as Tajima's D statistics were significant and negative (Table 4.4), rejecting the null hypothesis of population neutrality and supporting the notion of a recent population expansion. Non-significant results for Fu's F^* and D^* statistics also indicated population expansion as opposed to selection (Table 4.4). Finally, the mismatch distribution of pooled southern populations was not significantly different from a Poisson distribution and so was also consistent with population expansion (Fig. 4.3a; Avise et al. 1987).

For the combined northern population the pattern was not as clear. Although several common haplotypes were found, the parsimony network still approximated a star-shaped pattern, suggesting recent expansion (Fig. 4.2). Fu's F_S and Tajima's D statistics were not significant, and the hypothesis of a stable population could not be rejected (Table 4.4). However, several lines of evidence suggest recent population expansion. Although non-significant, Fu's F_S was negative (Table 4.4), which is an indication of expansion. In addition, non-significant Fu's F^* and D^* tests also generally indicate population expansion (Table 4.4). Lastly, the unimodal mismatch distribution pattern of the northern population was not significantly different from that expected under a sudden expansion model, and thus suggests recent population expansion (Fig. 4.3b; Avise et al. 1987).

The occurrence of several common haplotypes may also mean that there were several refugial populations north of the BMC that may have experienced less severe isolation than that seen in the southern populations. Several refugial populations with either occasional gene flow, or a less severe initial bottleneck could create a pattern consistent with the one found in this study.

Sequences from the BMC itself fall within the haplotype cluster of the southern populations, suggesting that Chowchillas recolonised the BMC from the south.

Table 4.4. Neutrality, diversity and expansion indices including expansion time estimates for Chowchilla mtDNA control region sequences. Significant values are indicated in bold (* $p < 0.05$, ** $p < 0.0001$). Patterns expected under selection or expansion are also given.

	North of BMC	South of BMC	Total population	Expected under Selection	Expected under Expansion
n	16	46	62		
No. unique haplotypes	6	8			
Haplotype diversity (h)	0.833	0.377	0.655		
Nucleotide diversity %	0.89	0.45		Low	Low
π	0.00449	0.00120	0.00726		
Sequence divergence			0.015		
Net Sequence divergence			0.011		
Tajima's D	0.20	-1.58*		Sign.	Sign.
Fu's Fs	-1.13	-6.11**		ns	Sign.
Fu and Li's F*	-1.76	-2.46		Sign.	ns
Fu and Li's D*	-1.74	-2.20		Sign.	ns
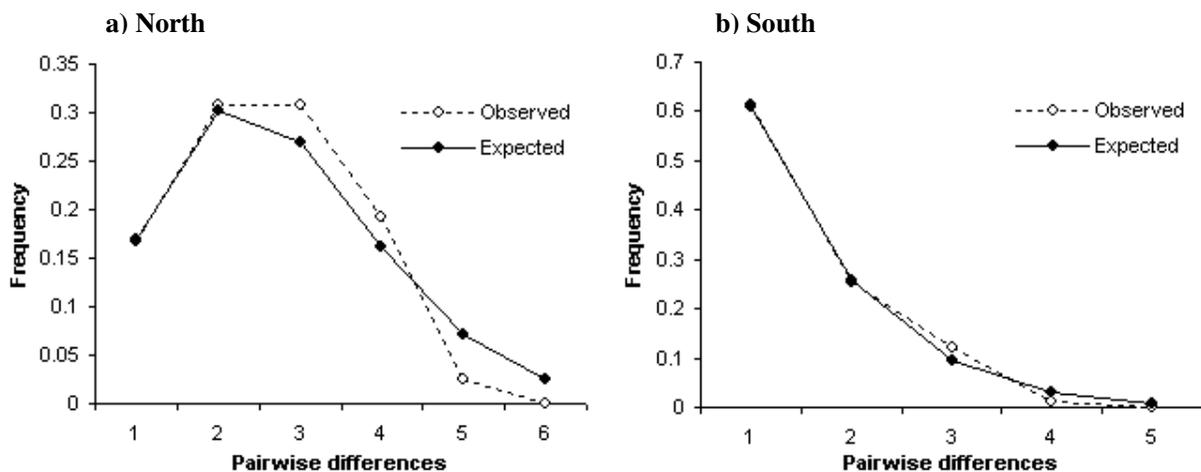
Figure 4.3. Observed and expected mismatch distribution for Chowchilla mtDNA control region sequences under the expectations of the sudden expansion model (Avise *et al.* 1987): a) populations north of the BMC, and b) pooled populations south of the BMC.

4.3.3 Divergence times

Estimated expansion times varied greatly depending on mutation rate and generation time. The most recent estimate at high mutation rates (20% per million years) and short generation time (1 year) dated population expansion at approximately 17 000 years bp, which coincides with the peak of the most recent glacial advance. However, slower mutation rates and longer generation times resulted in estimates of 50 000 to 600 000 years bp. All estimations, despite their high uncertainty, placed the population divergence well within the Pleistocene.

4.4   Discussion

This study is the first to clearly show distinctive morphological divergence across the BMC in a Wet Tropics endemic species. Chowchillas showed significant morphological divergence in absolute body size across the BMC regardless of sex, with northern birds being significantly smaller than southern birds (Table 4.1, Fig. 4.1a). Most other morphological characters examined were also smaller north of the BMC. Both males and females were lighter north of the BMC, and only northern males showed a reduction in relative weight (Table 4.1). In addition, this study is also the first to document the striking sexual size dimorphism in this species. With the exception of bill length, all morphological variables were significantly larger in males than females, irrespective of location (Table 4.1, Fig. 4.1a). Interestingly, despite strong morphological divergence across the BMC and between the sexes, absolute bill length remained stable across the whole population and among sexes (Fig. 4.1b).

I also found clear evidence of molecular genetic structuring across the BMC (Fig. 4.2). This structuring is consistent with previous phylogenetic studies including some birds, lizards and frogs (Joseph et al. 1995; Schneider et al. 1998; Schneider and Moritz 1999; Joseph 2001) and further confirms the presence of an old climatic barrier centred on the BMC. The magnitude of sequence divergence in the Chowchilla found across this barrier suggests that populations were isolated either side since the Pleistocene. Unfortunately, little is known about the Chowchilla's social system and generation time, and divergence time estimates themselves are fraught with uncertainty (García-Moreno 2004; Peterson 2006). Nevertheless, expansion time estimates clearly place Chowchilla population divergence well within the Pleistocene glaciations. It is possible that northern and southern populations could have split during the most recent glacial advance approximately 18 000 years bp, if control region mutation rates are very high (>20% per million years) and generation times very short. However, this is unlikely given the longevity, territoriality and social group living of Chowchillas, all of which point towards longer generation times and, hence, divergence times. Interestingly, divergence times estimated in this study are very different to Avise & Walker's (1998) estimate of 750 000 years, which was based on Joseph & Moritz's (1994) study on Chowchilla RFLP analyses and a mutation rate of 2% per million years. In a later study on mitochondrial *cytochrome b* divergence, Joseph & Moritz (1994) found slightly higher levels of net sequence divergence (2.4%) to the control region

divergence (1.5%) in this study. However, discrepancies in sequence divergence and divergence time estimates may be attributed to the very small sample sizes and distribution of both previous studies (5 & 11, respectively; Joseph and Moritz 1994; Joseph et al. 1995).

Although no further sub-structuring was found within the southern or northern populations, unimodal mismatch distributions and common shared haplotypes suggest recent population expansions to the south and possibly north of the BMC. In the southern population, these data also suggest recolonisation from a single refugial source. This scenario is consistent with previous findings and data interpretations for other Wet Tropics endemic fauna (Schneider *et al.* 1998). Following the final Pleistocene Glacial advance approximately 18 000 years bp, climatic conditions changed to cool and wet during the Holocene (7 500 – 6 000 years bp) promoting rainforest expansion. It is thought that during this time previously isolated populations expanded and recolonised the Wet Tropics beyond today's current rainforest distribution, including the now isolated Paluma ranges (Schneider *et al.* 1998). The current isolation of the Paluma Ranges is not apparent in my genetic data, as the broadly shared haplotype of the southern populations was also found in Paluma.

The haplotypes found within the BMC showed a closer affinity to the common southern haplotype and are distinctly different from the northern haplotypes. This strongly suggests that Chowchillas recolonised the BMC from the south, despite the fact that the samples were collected at the northern end of the BMC from locations having closer geographic affinity to the northern refugia. Morphologically, Chowchillas from the BMC also seem to be more similar to the southern populations, although sample sizes were small for this comparison, and further sampling is needed for confirmation. Interestingly, song from within the BMC was more similar to northern song in peak frequency, but intermediate between northern and southern song in bandwidth. It is possible that isolation-by-distance along the BMC or potentially different or marginal habitat may have influenced song within the BMC. However, more thorough genetic, morphological and song sampling within the BMC is needed to determine the processes creating opposing patterns of divergence in these traits.

The lack of variation in bill size is intriguing given the divergence found in all other morphological variables across the BMC. Two possibilities could explain this pattern. Firstly, bill size may have remained constant over time by chance, while smaller or larger body size was actively selected for through natural or sexual selection within the rainforest isolates. This would require differential selection pressures between northern and southern isolates as well as a break-down in allometry among characters, and contradicts the previous notion of relatively stable selective regimes in these rainforest isolates (Schneider and Moritz 1999). On the other hand, a more likely explanation is that while all other morphological characteristics were free to drift, bill size was under strong natural/stabilising selection. Variation in beak morphology within and between species has been shown to be strongly influenced by foraging strategies (e.g. Grant 1999), and the distinct lack of

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variation in bill size may be an indication of a very narrow feeding niche occupied by both sexes. Chowchillas feed on leaf litter invertebrates (Jansen 1993), and there are several other ground-feeding insectivorous and omnivorous bird species within the Chowchilla's range, encompassing a range of body sizes. Such interspecific competition may have put selective pressure on strict maintenance of the Chowchilla's feeding niche.

Chowchillas also show consistent patterns of sexual dimorphism in both size and plumage colour both north and south of the BMC, despite absolute size differences among lineages. Males are not only larger and heavier than females (Fig. 4.1a) but they also have a uniformly white chest and belly, while females have a distinct cinnamon chest and a white belly (see Plate 1). Sexual size dimorphism is commonly attributed to either differential niche utilisation by each sex, or intra-sexual competition (Andersson 1994). Both a lack of bill size differences between sexes and field observations suggest that male and female Chowchillas share the same feeding niche (Jansen 1993). This makes differential niche partitioning unlikely. Chowchillas are also very territorial, which provides an opportunity of male-male competition to operate. However, both sexes are involved in territorial defence, and for this hypothesis to be true, both sexes must defend unequally. No data are currently available to test this hypothesis. Social group living and reversed sexual colour dimorphism further blurs the clear differences in sex roles needed to explain size dimorphism using standard theoretical models.

Despite obvious, conventional sexual size dimorphism, Chowchillas show reversed sexual plumage dimorphism with females sporting a bright orange chest. Carotenoid-based orange ornamentation is considered to be an honest, condition-dependent signal that plays an important role in (traditionally female) mate choice (Endler 1980; Hill 1991). The origin and function of female ornamentation in species with conventional sex-roles, on the other hand, remains unclear, but is assumed to be associated with sexual selection through male mate choice or female resource competition (Amundsen 2000). Therefore, while the exact evolutionary processes responsible for sex-specific differences in Chowchilla remain unclear, current theory suggests the consistent patterns of dimorphism among lineages must revolve around a complex mating system that involves elements of sexual selection and/or intra-sexual competition.

Although differential selective pressures could have promoted divergence in morphology either side of the BMC, this seems unlikely due to the similarity of the rainforest habitats that northern and southern populations inhabit. Thus, based on previous interpretations of similar molecular data sets, my findings suggest that vicariant isolation has had a strong effect on morphological as well as molecular genetic divergence in the Chowchilla. Bottleneck effects during the original rainforest contraction, as well as drift during isolation are likely to have shaped some components of Chowchilla morphology. However, contrasting patterns of conventional sexual size dimorphism, reversed sexual plumage dimorphism and bill morphology also suggest that both sexual and natural selection have been

important in establishing final levels of diversity. Thus, contrary to current perception, morphological divergence across the BMC demonstrates that vicariant isolation is indeed sufficient to produce phenotypic divergence in a rainforest system. Thus, patterns of morphological variation or lack thereof provide important insights into the relative importance of the different evolutionary processes. The genetic and morphological divergence presented in this Chapter, as well as evidence of significant song divergence across the BMC (Chapter 2), support the division of this taxon into two sub-species as suggested by Schodde & Mason (1999).

The results from this and previous Chapters lead to several very intriguing questions: Do Chowchillas recognise and discriminate between different song variants? Do Chowchillas respond more strongly to neighbours' or strangers' song? At what scale do they discriminate between song variants? Do they still recognise song from the different subspecies? And following on from this question, has song diverged sufficiently enough to create a pre-mating barrier to gene flow between the northern and the southern populations? These questions form the third aim of this thesis and will be addressed in the following Chapter.

CHAPTER 5   The Evolutionary Consequences of Song Variation

Publication arising:

Koetz, A. H., Westcott, D. A. & Congdon, B. C. (2007). Song discrimination within and between subspecies reveals function & consequences of song dialects. *Behavioral Ecology (in review).*

5.1   Introduction

As the previous chapters have revealed, historical vicariant isolation in Pleistocene refugia and across the older BMC has resulted in strikingly concordant patterns of divergence in song (Chapter 2), morphology and molecular genetics (Chapter 4). These results support the notion of sub-speciation in the Chowchilla. In addition, small-scale social processes have also resulted in a mosaic distribution of song dialects with a sharp decline in song similarity over very short distances. This suggests a strongly social (territorial) function of song dialects (Chapter 3). These patterns lead to the important question: Have *O. s. melasmenus* and *O. s. spaldingii* diverged sufficiently for pre- or post-mating barriers to gene flow to exist between the subspecies, and can a cultural character trait involved primarily in territorial defence provide such a barrier?

5.1.1 Large-scale song discrimination

According to the Biological Species Concept, reproductive isolation is the defining attribute of a species (Ernst Mayr 1942 in Coyne and Orr 2004). Reproductive isolation is ensured by isolating barriers, which may be pre-mating, post-mating or post-zygotic (Coyne and Orr 2004). However, how reproductive isolation is achieved and then maintained upon secondary contact is still unclear. Diverging mating signals are effective isolating barriers between different species (West-Eberhard 1983; Coyne and Orr 2004). Therefore, divergence in mating signals is thought to potentially be a strong driver of speciation (Marler and Tamura 1962; Baker 1975; Irwin and Price 1999; Uy and Borgia 2000; Slabbekoorn and Smith 2002a; Seddon and Tobias 2007). Bird song is an important mating signal, and so divergence in bird song may play an important role in causing, maintaining and/or reinforcing reproductive isolation in the process of avian speciation. Thus, Baker (1982) postulated that song learning and song divergence may have contributed to the immense species diversity of oscine birds. Following is a review of how divergence in bird song may lead to reproductive isolation as well as the evidence for or against this notion.

As introduced in Chapter 2, due to the inaccuracies of the learning process some level of spatial variation in song is inevitable (Slater 1989). Differences in song among populations may be enhanced through the processes of cultural drift in isolation or across large distances, founder- or bottleneck effects, and/or through the influence of natural selection. On secondary contact, such divergence in

song could potentially lead to a behavioural, pre-mating barrier to gene flow, if songs have diverged enough that communication is no longer effective and assortative mating ensues. However, whether such divergence in song, a behavioural, cultural trait, may then lead to assortative mating and reproductive isolation is still highly debated (Grant and Grant 1997b; Irwin and Price 1999; Slabbekoorn and Smith 2002a).

Evidence for this process has been found where sympatric species show some level of hybridisation. A classic example is that of Darwin's finches, where song divergence that occurred in isolation resulted in a behavioural pre-mating barrier to gene flow on secondary contact (Grant and Grant 1997b; Grant and Grant 1997a). Similar evidence was also found in hybridising Lazuli and Indigo buntings (*Passerina amoena* and *P. cyanea*) (Baker and Boylan 1999).

Less direct evidence comes from the study of a ring species, the Greenish warbler, *Phsylloscopus trochiloides*. This species expanded northwards either side of the Tibetan Plateau, forming a ring of interbreeding populations. However, where the populations meet north of the Plateau, reproductive isolation occurs (Irwin *et al.* 2001). Playback experiments showed that individuals from the two populations at this interface do not respond to the song of the alternative population. Thus, the accumulation of differences in song complexity and structure in allopatry is thought to have led to a behavioural, pre-mating barrier to gene flow upon secondary contact at the endpoints of the ring (Irwin *et al.* 2001).

Although clear and consistent evidence for assortative mating based on intraspecific variation is scarce (Slabbekoorn and Smith 2002a), these examples demonstrate that divergence in song can indeed act as a behavioural pre-mating barrier to gene flow that may ultimately cause speciation. Thus, testing whether and to what extent birds discriminate between song variants from clearly divergent populations of subspecies is a crucial step in determining whether large-scale song divergence may be sufficient in creating a behavioural pre-mating barrier on secondary contact.

5.1.2 Small-scale song discrimination

Birds may also discriminate between song variants within populations. At this smaller, local scale research often focuses on the functional significance of geographic variation such as neighbour *vs.* stranger or local *vs.* foreign song discrimination (e.g. Payne et al. 1991; Searcy et al. 1997; Wright and Dorin 2001; Nelson and Soha 2004a; Nelson and Soha 2004b; Hardouin et al. 2006). Whether or not birds respond to local or foreign variants, and how strongly, depends on the threat perception of neighbours *vs.* strangers (Temeles 1994), and also on dispersal distances and the likelihood of ever encountering a different dialect (Searcy *et al.* 2002). Although some species perceive strangers as a larger threat than established neighbours (also referred to as the 'dear-enemy-effect', reviewed in Temeles 1994), most territorial birds perceive neighbours as a bigger threat and, hence, respond more

strongly to local song variants than to foreign ones (Searcy et al. 1997; Searcy et al. 2002; Nelson and Soha 2004a). A review of species with and without the dear-enemy-effect indicates that whether or not neighbours are perceived as a greater threat strongly depends on the territorial system; species generally respond more strongly to neighbours when defending feeding resources (Temeles 1994). Whether or not song variation affects communication between individuals also depends on the geographic scale at which song changes, as well as on how far birds disperse within their lifetime (Searcy *et al.* 2002). In particular, song variation is likely to play an important role in species with abrupt dialect boundaries where individuals of different dialects would be expected to interact often. In such systems variation in song is thought to play an important part in territorial defence and mate choice (Searcy *et al.* 2002).

Discrimination of song variants within a bird's dispersal distance (local *vs.* increasingly foreign) can give an insight into the function of dialects and their discrimination. On the other hand, discrimination of songs from more distant, isolated populations and/or from different subspecies is crucial in determining whether large-scale song divergence may be sufficient in creating a behavioural pre-mating barrier on secondary contact. Therefore, testing whether and to what extent birds discriminate between song variants at several different scales is crucial in determining the behavioural significance of song variation, and, in turn, the functional and evolutionary importance of such variation (Falls 1992).

Few studies examine both large-scale song divergence and its potential as a barrier for gene flow, and smaller-scale song variation and discrimination within local subpopulations. In addition, most studies on song discrimination have been conducted on species in the temperate, northern hemisphere. Temperate species generally exhibit the conventional traits of predominantly male song used for resource acquisition and defence, and female mate choice based on a male's song and his resources (Catchpole and Slater 1995). There is a distinct lack of research focussing on tropical species that often do not adhere to these classical roles (Stutchbury and Morton 2001), particularly cooperative and/or group-living birds that defend stable territories and sing year-round. Many such tropical systems also show females to be strongly involved in singing and territorial defence (Stutchbury and Morton 2001; Fedy and Stutchbury 2005). Thus, such systems are invaluable in providing new and challenging insights into the function and evolutionary importance of song divergence.

In the present study, I conducted playback experiments on the Chowchilla in order to test the perception of different song variants at a number of spatial scales in this species. The Chowchilla is of particular interest for a study on song discrimination for several reasons. In addition to having exceptional geographic variation in song at small and large spatial scales and across historically isolated populations, both male and female Chowchillas engage in territorial defence equally using the same song. Furthermore, the Chowchilla has a single-song repertoire, with several groups in a

neighbourhood sharing this single song type (Chapter 2 & 3). This removes the potentially confounding aspect of individuals having large and variable song repertoires. In addition, having only one type of song also means that Chowchilla song most likely functions as a mate choice and/or advertisement signal involved in sexual selection in addition to territorial defence. These attributes make the Chowchilla exceptionally suitable for a study on song discrimination across several spatial scales. In particular, this system allows me to test for discrimination of within-subspecies variation at several scales, as well as for possible discrimination of songs from the different subspecies. The former allows an insight into the possible function of such variation, whereas the latter allows a test of whether the divergence in song found across the BMC may function as a potential pre-mating barrier to gene flow between the two subspecies.

The aims of this Chapter are to:
 1) determine whether Chowchillas (*O. s. spaldingii*) show differential responses to local and increasingly distant song dialects, including song variants from historically isolated populations of the same subspecies; and
 2) test whether Chowchillas recognise and discriminate between songs of historically isolated populations from the different subspecies (*O. s. melasmenus*).

5.2   Methods

5.2.1 Study sites & recordings

Playback experiments were conducted at three different locations within the Lamb Range on the Atherton Tablelands (Fig. 5.1). At each location, playback was conducted to two resident groups of Chowchillas separated by approximately 1.5km, which ensured independence of these groups. Songs used in this experiment were recorded across the Chowchillas' range between April and November 2004 and August and November 2005 as outlined in Chapter 2. Six representative songs from each of the six Lamb Range test-groups as well as from three additional refugia were chosen from these spectrograms (see below).

5.2.2 Playback design

The response of the southern subspecies of the Chowchilla (*O. s. spaldingii*) to local and foreign song dialects was tested by imitating a territorial intrusion using playback. Playback trials were conducted between 7:30 and 11:30am, October 3 - 28, 2005. Trials were conducted from the approximate edge of a group's territory and aimed towards the centre of the territory. The response of all birds within the resident group was noted continuously by two observers, stationed at 10m either side of the speaker.

The song dialects tested included songs from different spatial scales, different levels of historical isolation, as well as from the different subspecies. At the smaller scale, test dialects included songs

from three locations within the Lamb Range refuge (Davies Creek, DC; Kauri Creek, KC; Clohesy Fig, CF; Fig. 5.1). At the medium scale, test dialects included songs from within the same subspecies (*O. s. spaldingii*) but from a historically isolated population (Walter Hill, WH; Fig. 5.1). Lastly, at the largest scale, test dialects included songs from the different subspecies (*O. s. melasmenus*), which was historically isolated across the BMC as well as within northern refugia. Songs from two alternative northern refugia were tested (Thornton Uplands, TU, and Carbine Uplands, CU; Fig. 5.1).
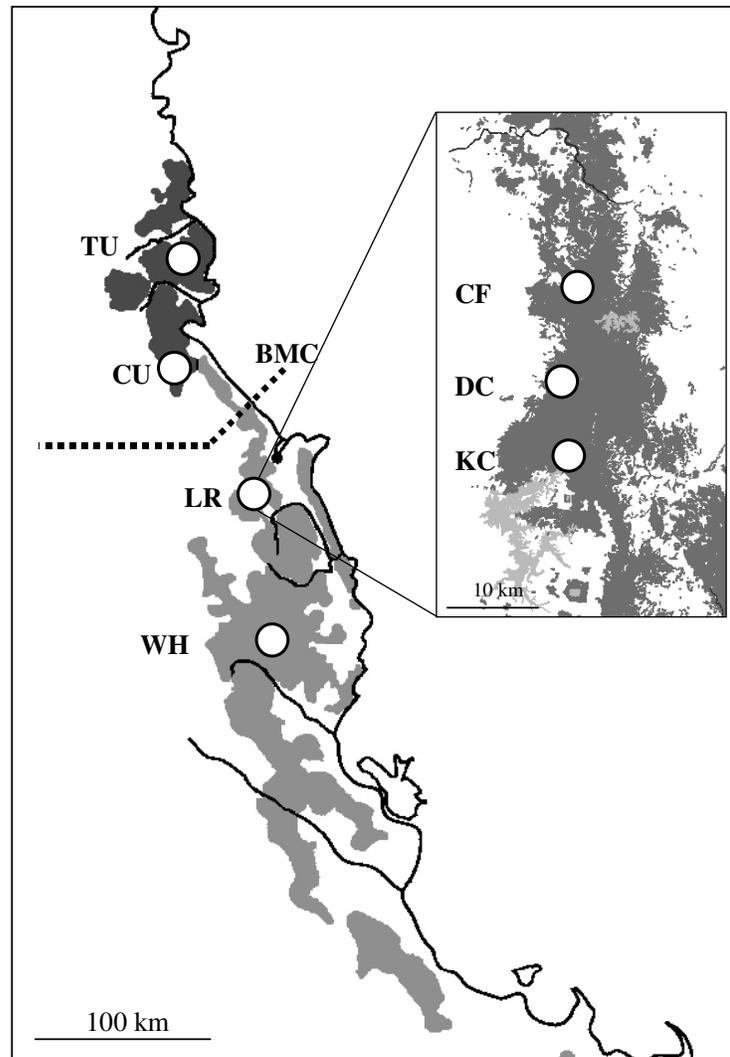
Figure 5.1. Map of the current distribution of the Wet Tropics World Heritage rainforest. Dark grey indicates the distribution of *Orthonyx spaldingii melasmenus* north of the BMC, lighter grey indicates the distribution of *O. s. spaldingii* south of the BMC. Locations of test-dialects across the Chowchilla's range are indicated by white circles. Insert depicts a map of the Lamb Range and the position of the three playback locations (CF, DC & KC).

Each of the six test-groups was tested with the local dialect ('local'), the dialect of the second group at that location ('other site' – average distance 1.5km), one dialect each from the other two locations within Lamb Range ('other location' – average distance 15km), as well as a dialect from the different refuge within the same subspecies ('other refuge S' – average distance 70km) and two refugia north of the BMC representing dialects of the different subspecies, *O. s. melasmenus* ('other refuge CU' and 'other refuge TU' – average distance 60 and 110km, respectively). Thus, seven trials were conducted with each group, including dialects of increasing distances within the same subspecies, as well as dialects from the different subspecies.

The order of sites visited and dialects played was established using a Latin square design. Thus, each group was visited every two to six days over a four-week period. Pseudoreplication is a common and persisting problem in playback studies (McGregor et al. 1992; McGregor 2000; Kroodsma et al. 2001). In order to avoid it, I used the following procedure. For each of the seven dialects tested, I selected six representative songs (one each to be played to the six test-groups). These songs were of high quality and low background noise and were sung by different individuals (males only). If songs from different individuals were not available, different renditions of the same song of the same individual were used. Thus, no single song stimulus was played more than once. This design was possible because all members from a single cooperatively breeding group sang identical songs.

Song stimuli were played from a laptop computer (Toshiba Tecra Intel Celeron M), using Avisoft SASLab Pro (Specht 2005), and a self-built speaker box (henceforth simply called 'speaker') consisting of an amplifier (Coustic Power Logic Component AMP 108) and two front-facing speakers (Pioneer TS-G1050). The speaker was set at ground level, and the sound level was adjusted to 65 db (A) at 10 meters (m) from the speaker, measured with a Radio Shack sound level meter. This sound level approximates the actual sound level of singing Chowchillas at 10 m (personal observation). To estimate distances in the forest, flagging tape was placed on trees approximately 2 m above ground in 5-m intervals, along two to four transects away from the speaker and up to 25 m depending on forest density and visibility.

5.2.3 Test stimuli

Playback stimuli were constructed to represent the natural song rate of a single Chowchilla singing, which approximates one song every 1-2 seconds, or 6-12 songs per minute (personal observation). Hence, each song was repeated every 1-1.5 seconds for one minute (equalling 6-10 songs per minute depending on song length), followed by 10s of silence. This was repeated three times, resulting in a 3.5-minute set. This set of song stimuli was repeated up to 5 times with a 1-minute silent interval between sets during each trial.

The trials began by observing any Chowchilla activity (visible or audible) for 2 minutes prior to playback. The playback stimuli were then started and repeated up to 5 times or until birds started to sing, in which case the playback was stopped. This was followed by one to two minutes of interactive playback to simulate a territorial encounter. After this interactive playback, the tape was stopped and the birds' response observed for another 5 minutes. If birds did not respond to the test stimulus, the playback trial was followed by playback of the local dialect as a control, using the same design. If birds responded to this control, I assumed that birds were capable of responding to the test stimulus but chose not to, and thus their negative response was counted for the analysis. However, if birds did not respond to this control, they were assumed to be out of hearing distance and the trial was not counted but repeated at a later date. If neighbouring groups responded before the target group, the trial was not counted and repeated again at a later date.

5.2.4 Response measures

During the 2-minute pre-playback and during and after playback, any activity observed or heard was noted along with the time at which this occurred for calculation of latencies. Also noted were distance from the speaker and, where possible, the sex that responded first and/or approached the closest.

Two types of response measures were used for analysis. I used two different types of response measures: (1) the number of trials in which groups responded by approaching and/or singing, as an absolute measure of whether or not groups responded (qualitative response measure); and (2) approach and song latencies were calculated as measures of the strength of response (quantitative response measures). Due to relatively low visibility in the forest, time to approach was loosely defined as the time until a bird was first seen or heard, including any Chowchilla-specific noises (e.g. scratching, chuckle or growl calls). However, the Chowchillas' loud song is exceptionally audible and easily located, and thus song latency was easily determined. Additional response variables included the closest distance to the loudspeaker that any member of a group approached, and, where visibility allowed, the sex of the bird that approached first and/or the closest.

5.2.5 Statistical Analyses

Whether birds responded significantly more or less to different song variants was analysed using a derivative of Fisher's Exact test (Fisher-Freeman-Halton test), which calculates all possible outcomes of data arranged in an r x c contingency table. Thus, this test calculates the true (exact) distribution of the test statistic and tests the null hypothesis of independence. Exact tests are particularly suitable for small, sparse and unbalanced data that do not adhere to the assumptions of a χ^2-square test (Freeman and Halton 1951; Agresti 2001). Response data (song and approach) were arranged in contingency tables of 2 x 4 (response/no response *vs.* four test stimuli from the same subspecies only) and 2 x 6 (response/no response *vs.* all six test stimuli, including those from the different subspecies) and analysed using StatXact (Version 7, www.cytel.com).

To analyse response latencies, I used inverted song and approach latencies in order to include trials in which birds did not respond to the test stimulus but did to the control (local dialect). In this case, latency was set at zero. Both approach and song latency were tested in a between-subjects repeated measures analysis of variance (ANOVA) model, with dialect stimulus as the within-subject effect and location as the between-subject (block) effect. Significant results for this analysis are presented as mean latencies ± 2 S.E. for interpretation only.

Sample sizes correspond to the number of stimulus sets (dialects) rather than individual groups in order to avoid pseudoreplication.

5.3   Results

A local Chowchilla group typically responded to local playback by quickly running toward the loudspeaker, passing it and then stopping at some distance from the speaker. Individuals then turned around for another running pass, or for a slow approach while engaging in territorial song. Some groups commenced singing before closing in, or sometimes after a first run past. Responses to foreign dialects varied anywhere from a full territorial response to no response at all.

In 37 out of 40 trials (92.5%) Chowchillas responded by approaching close enough to be seen or heard (< 25 meters), irrespective of the dialect played (Fig. 5.2). However, Chowchillas did not approach significantly more or less to different test stimuli, either within their own subspecies (Exact statistic = 1.984, $p = 0.807$), or for all test dialects (Exact statistic = 3.09, $p = 0.851$).

In 31 out of 40 trials (77.5%) Chowchillas responded by engaging in territorial song. Chowchillas responded by singing upon hearing the local dialect in all local dialect trials. However, they responded in a decreasing number of trials to dialects from farther away (Fig. 5.2). This outcome was significant (Exact statistic = 9.755, $p = 0.015$) and indicated that within the southern subspecies the intensity of response decreased with playback song from increasing distances away.

When comparing these results with the responses to dialects of the alternative subspecies, it becomes clear that test groups responded more often to dialects from the northern refugia (different subspecies) than they did to dialects from their own subspecies (southern refuge). Groups responded four and three times more often to subspecies song from 60km and 110km distant, respectively, than they did to dialects from the most distant within-subspecies dialect (Fig. 5.2). When responses to all dialect types (both within and between subspecies) were included in a single analysis, the pattern becomes non-significant (Exact statistic = 10.22, $p = 0.053$), although marginally so.
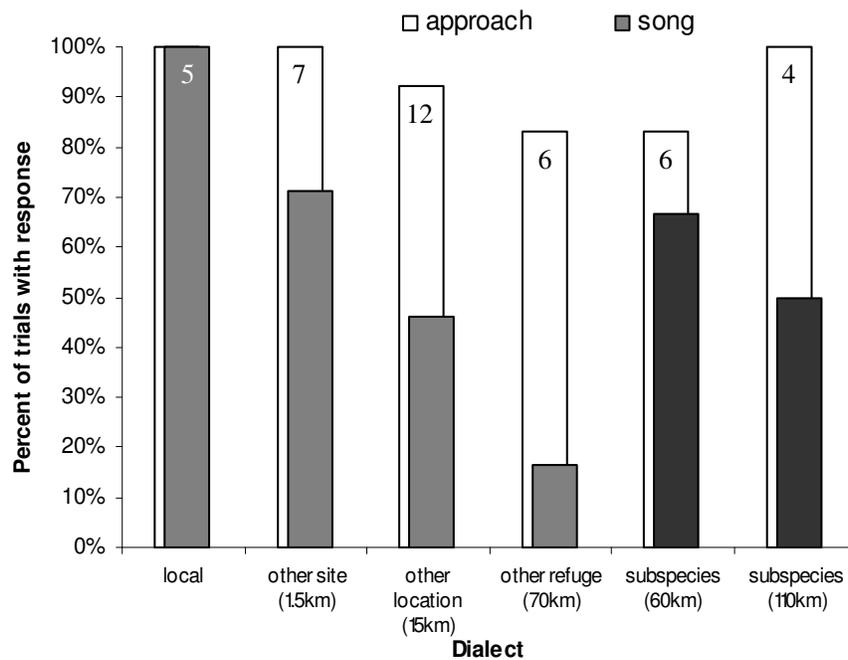
Figure 5.2. Percentage of trials in which Chowchillas responded by approaching (white bars) and singing (grey bars) to different dialect stimuli. Light grey bars indicate test dialects from within the same subspecies, whereas dark grey bars indicate test dialects from the different subspecies (north of the Black Mountain Corridor). Numbers in the bars denote sample sizes.

Results for the measures of response strength (inverse approach latency, inverse song latency and closest approach distance, m) for each dialect stimulus are given in Table 5.1. The Repeated Measures ANOVA showed that approach latency was not significantly different between dialects within or between blocks ($F_{4,6}$ = 0.511, p = 0.73 and $F_{2,6}$ = 0.086, p = 0.921, respectively). However, inverse song latency decreased significantly with dialects from increasing distances ($F_{4,6}$ = 4.435, p = 0.02) irrespective of the block/location ($F_{2,6}$ = 1.513, p = 0.351) and without an interaction effect ($F_{4,6}$ = 1.217, p = 0.366). Thus, Chowchillas responded significantly quicker to the local dialect than to any other (Fig. 5.3; Table 5.1). In addition, groups showed the quickest response to the most similar (local) dialect (Fig. 5.3; Table 5.1). There was no difference in song latency between dialects from the next site and those farther away despite the greater similarity of next-site dialects (Fig. 5.3). There was also no difference in approach distance between dialects ($F_{4,35}$ = 1.411, p = 0.254), although birds tended to come closer (~ 5 m) upon hearing local dialect than hearing other dialects (~ 9 – 12 m; Table 5.1).
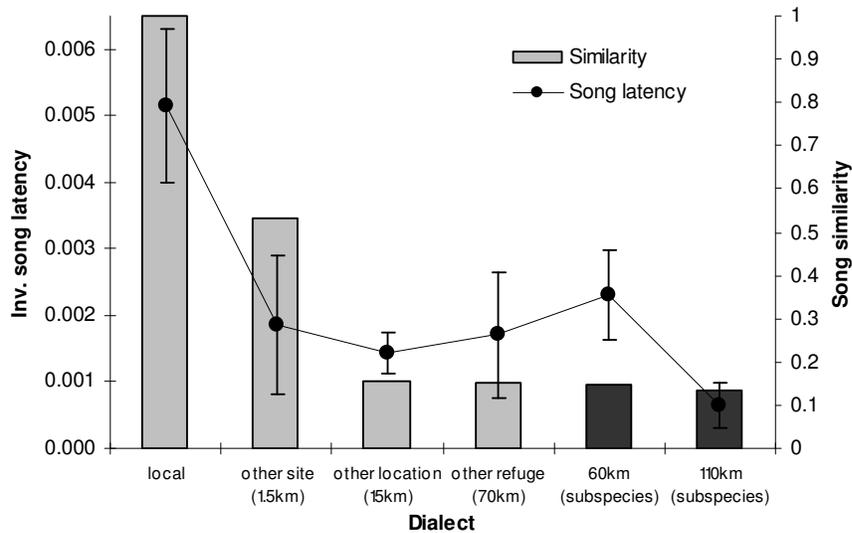
Figure 5.3. Inverse time to response by song (song latency ± 2S.E.) and similarity of the song dialect to the test groups' local song (song similarity – grey bars). Light grey bars indicate similarity of test dialects from within the same subspecies, whereas dark grey bars indicate similarity of test dialects between subspecies.

Table 5.1. Average response strength (± SD), measured as inverse approach latency, inverse song latency and closest approach distance (m) for each dialect stimulus. Test dialects 'local', 'other site', 'other location' and 'other refuge S' are all from within the same subspecies (*O. sp. spaldingii*); test dialect 'other refuge CU' and 'other refuge TU' are from the different subspecies (*O. s. melasmenus*).

Dialect stimulus	Subspecies	Inverse approach latency		Inverse song latency		Closest approach distance (m)	
		Mean	± SD	Mean	± SD	Mean	± SD
Local		0.0072	±0.0040	0.0051	±0.0028	4.8	±0.4
Other site	*O. s.*	0.0287	±0.0415	0.0019	±0.0025	12.0	±9.1
Other location	*spaldingii*	0.0075	±0.0061	0.0014	±0.0008	9.3	±5.5
Other refuge S		0.0088	±0.0073	0.0017	±0.0021	12.6	±3.0
Other refuge CU	*O. s.*	0.0074	±0.0058	0.0023	±0.0017	9.1	±2.7
Other refuge TU	*melasmenus*	0.0077	±0.0070	0.0006	±0.0006	9.8	±3.5

In a small number of trials it was possible to determine the sex that approached first ($n = 16$) or the closest ($n = 18$) for more than two trials per dialect stimulus. Due to the small sample size, no statistical tests were performed. However, results suggest that males tended to approach first and closest more often than females in response to local and next-site dialects (Fig. 5.4a & b), while females tended to approach first and closest much more often than males in response to dialects from different refugia (Fig. 5.4a & b).

As a control, the effect of random or non-specific noise and of songs from a number of different species on Chowchilla responses was tested before commencing this experiment. Birds did not respond to any noise or control song played.
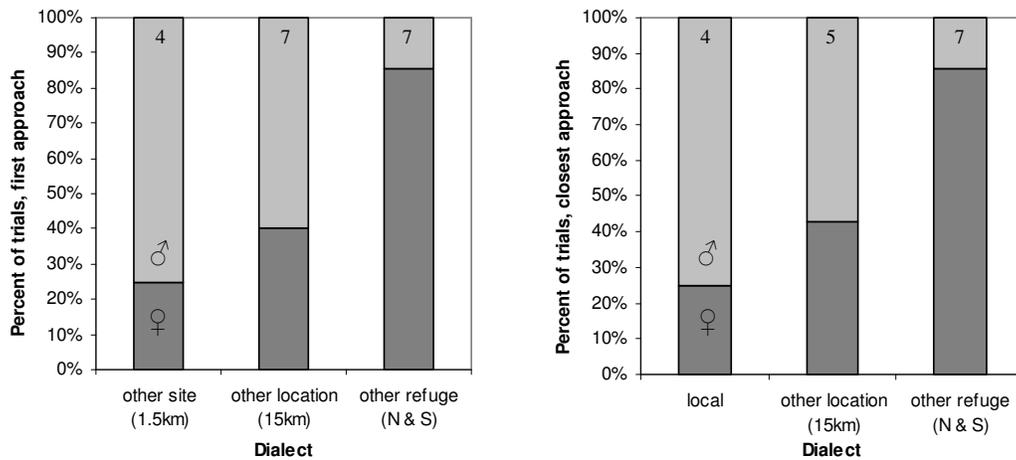
Figure 5.4. Relative response of the sexes to different dialects. Bars indicate the percent of males (light grey) or females (dark grey) responding by approaching first (a) or closest (b); sample sizes for each dialect are indicated.

5.4 Discussion

These results suggest that Chowchillas recognise their species-specific song irrespective of its geographic or sub-specific origin (Fig. 5.2). Nevertheless, Chowchillas discriminate between local and foreign songs within their own subspecies, shown by the declining percentage of trials eliciting song responses with increasingly distant dialects (Fig. 5.2), as well as by the sharp decline in inverse song latency with increasingly distant dialects (Fig. 5.3). However, there was a surprisingly high number of trials in which birds responded to dialects of the different subspecies. The closest dialect of the different subspecies (CU) is at a comparable distance to the most distant within-subspecies dialect (WH), yet groups responded to the dialect of the different subspecies four times more often. Despite

this high occurrence of singing responses, song latency was no different to those of foreign within-subspecies dialects.

5.4.1 Small scale implications (local vs. distant dialects)

The pattern of decreasing occurrence and strength of responses with increasing distance for within-subspecific song is surprisingly consistent with the pattern of song element sharing found in this species (Chapter 3). Song element sharing decreased sharply with increasing distance, with a particularly distinct drop in similarity between neighbouring (local) groups and groups at the next site (~1 km away; see Chapter 3). These geographic scales are directly comparable with those in this playback study. Thus, the distinct and significant drop in response strength between local dialects and other-site dialects (1-2 km away) corresponds directly with the distinct drop in song similarity. This strongly suggests that song element composition and sharing plays an important role in song discrimination in the Chowchilla. Most groups approach any dialect played, indicating species-specific song recognition. However, the time to song (response strength) following this initial approach (if it follows) differs depending on the distance and, hence, the similarity of the dialect.

Chowchillas' response to local *vs.* foreign song falls within the general pattern often found in territorial songbirds, i.e. territorial birds (usually males) respond more strongly to local song variants than to foreign ones (e.g. Searcy et al. 1997; Wright and Dorin 2001; Leader et al. 2002; Searcy et al. 2002; Nelson and Soha 2004a). Thus, this study extends the generality of this pattern to tropical, group-living birds in which both males and females sing.

Two primary hypotheses have been proposed to explain stronger territorial responses to local *vs.* foreign song variants in songbirds: the 'recognition' hypothesis and the 'relative threat' hypothesis. The 'recognition' hypothesis proposes that the response pattern is a by-product of species song recognition, i.e. that birds will respond most strongly to a song variant that matches an individual's internal representation of its species' standard song most closely (Dabelsteen and Pedersen 1992; Nelson 1998). Under this model, a local bird is expected to respond progressively less to songs that are increasingly dissimilar, to the extent that foreign dialects are not recognised as species-specific. However, this hypothesis is not particularly suitable to species in which birds readily disperse across dialect boundaries (Wright and Dorin 2001). The 'recognition' hypothesis does not explain the differential response strength in the Chowchilla, due to the fact that in the majority of trials Chowchillas did approach any dialect played, despite much lower similarity of foreign song. Furthermore, Chowchillas sang surprisingly often in response to dialects of the alternative subspecies, which represent the lowest song similarity. Therefore, a stronger response to local song variants is unlikely to be a non-functional by-product of species recognition. Such a response pattern seems better explained by a territorial function as proposed in the 'relative threat' hypothesis.

The alternative 'relative threat' hypothesis suggests that local song variants elicit stronger responses because local birds (neighbours) singing the same or similar song represent a much greater threat to an individual's territory and resources than a foreign bird singing dissimilar song (Rothstein and Fleischer 1987; Temeles 1994). This higher threat level is thought to exist because birds singing the local song are more likely to display greater local experience and adaptation, and thus have a better chance at usurping a territory. Under this model, dispersing birds would be under selection to learn local dialects upon settling in order to have a chance at gaining a territory and mates. The spatial pattern of small-scale song element sharing in the Chowchilla suggests such post-dispersal song learning (Chapter 3). In addition, the 'relative threat' hypothesis seems to apply well to the response patterns found in this study.

5.4.2 Medium scale implications (within-subspecies dialects)

A decline in song similarity over distance is not surprising as this pattern would be expected through the processes of drift and/or selection, whether songs are learned or genetically determined (Searcy *et al.* 2002). However, few studies have tested for differential responses to dialects from increasing distances away (Searcy *et al.* 2002). Previous studies that did include several levels of spatial scale in their playback design showed similar patterns of declining responses across larger distances (Wright and Dorin 2001; Searcy et al. 2002; Vehrencamp et al. 2003). However, tentative conclusions about the function of this pattern varied, including greater familiarity with local songs (Vehrencamp *et al.* 2003), neighbours posing a greater threat (Wright and Dorin 2001), and female preference for similar song as an indicator of male song learning ability (Searcy *et al.* 2002).

The question then is: How are mosaic-type dialects maintained, if territorial defence is the most important function of song in the Chowchilla? If all birds shared the same song, the message to stay out of one's territory would be clear to everyone, to local and foreign birds. Thus, the occurrence and maintenance of strong song variation in this species may be a non-functional by-product of its learning and dispersal strategies. The development of mosaic-type dialects as a function of learning and dispersal is possible, as has been shown by modelling previously(Goodfellow and Slater 1986; Ellers and Slabbekoorn 2003). If dialects were indeed non-functional in the Chowchilla, it would explain why Chowchillas approach all dialects played. However, it would not explain the differential response in singing and song latency to increasingly different dialects.

Alternatively, there may be a function to being different, rather than being the same. If all birds sang the same song, strangers and neighbours alike would attract the same aggressive territorial attack. Given the complex social behaviour of Chowchillas, including the high probability of delayed dispersal and cooperative breeding, it is a possibility that strangers are not regarded as a threat by a local group. Rather, they may be seen as potential helpers or even mates, which may be a mechanism to reduce inbreeding. The tendency of females to approach first and closest to more distant dialects

would support this. In addition, territory size has been linked with group size in the Chowchilla previously (Jansen 1999), so attracting and adopting non-local birds may enable a group to expand its territory. However, given the uncertainty of the Chowchilla's mating system, this possibility remains speculative.

5.4.3 Large scale implications (between sub-species dialects)

The high occurrence of both approach and song responses of *O. s. spaldingii* to dialects from *O. s. melasmenus* leads to the conclusion that song of the two subspecies has not diverged sufficiently enough to create an effective pre-mating barrier to gene flow. This result is surprising in two ways. Firstly, distinct divergence in song spectrotemporal, morphological and molecular genetic features between the subspecies (Chapters 2 – 4) led to the expectation that northern and southern populations had diverged sufficiently to respond less (or not at all) to dialects of the different subspecies. Nevertheless, song of the different subspecies was clearly recognised.

Secondly, dialects of the different subspecies elicited territorial song in a much higher proportion of trials than did the most distant dialect from the same subspecies. This is despite the fact that the farthest within-subspecies dialect (WH) is at a comparable distance with the closer between-subspecies dialect (CU) or about half as far as the second between-subspecies dialect (TU). On the other hand, song latencies were comparable to those of more distant within-subspecies dialects.

Given the clear differences in song characteristics between the two subspecies (Chapter 2), they must still share certain song features that are perceived as similar enough to elicit approach and in some cases full territorial responses. Two possible reasons could account for this. Firstly, song from the different subspecies may be similar enough to recognise as the same species, but dissimilar and novel enough to arouse curiosity in the local birds. This could be linked to the Chowchilla's dispersal distance and the likelihood of ever hearing such a distinctly different dialect. However, this would not explain the very low response levels upon hearing dialects of the same subspecies from a different refuge (WH).

Alternatively, it is potentially possible that songs diverged by random drift in such a way that northern songs have a higher proportion of song features that southern birds use for similarity (and hence threat) assessment. Northern songs are characterised by a much larger proportion of short, warbled elements of low bandwidths than southern songs (Fig. 2.2 & 3.2). These elements appear to be more prone to degradation, as it is mainly the louder, frequency-attenuated (whip-like) elements of broad bandwidth that can be heard at larger distances (personal observation). Thus, if the warbled elements are used for short-distance communication and important in similarity and/or threat identification, an increase in these components in one subspecies could elicit unexpectedly high behavioural responses

in the other subspecies. If this hypothesis was true, conducting similar playback experiments in the northern refugia using southern songs should elicit quite different patterns of responses.

These results suggest that under the Biological Species Concept, the two subspecies could be considered as one species, due to the fact that Chowchillas recognised and approached all dialects played. Although there is no direct evidence yet that links Chowchilla territorial song with mate choice, given that Chowchillas only sing a single song type it is more than likely that this song also functions as a mate choice and advertisement signal. Chowchilla song is superiorly adapted for long-range communication in dense forest (Richards and Wiley 1980; Endler 1992; Ryan and Kime 2002), and so eavesdropping may occur, enabling mate assessment and choice during the dawn chorus or territorial encounters. Thus, recognition of foreign dialects as species-specific means that a pre-mating barrier through song variation is unlikely. This stands in stark contrast to the morphological and molecular genetic divergence found between the subspecies. However, phylogenetic and biological species concepts can yield opposing results given the fundamentally different markers used. In addition, it has been shown previously that some sympatric species do not interbreed due to a strong pre-mating barrier based on song (Grant and Grant 1997b; Grant and Grant 1997a), whereas some genetically distinct, allopatric species (e.g. two lineages of warbler finches, *Certhidea olivacea* and *C. fusca*) may still respond to playback of each other' song, i.e. a pre-mating barrier is lacking (Grant and Grant 2002a). This suggests that divergence in song and song discrimination may be stronger in genetically similar, sympatric species due to reinforcement during the early stages of speciation (Haavie *et al.* 2004; Hoskin *et al.* 2005). On the other hand, allopatric species that naturally would not meet may not be under such strong selection. To test this hypothesis, further playback experiments within and across the zone of secondary contact of northern and southern Chowchilla populations are necessary.

Although it is thought that northern and southern Chowchilla populations are currently connected across most of the Wet Tropics, it is not known whether the two subspecies are in fact in contact across the BMC. Chowchillas caught at the northern end of the BMC were genetically more similar to southern birds, which could mean that northern birds have not dispersed into the BMC. However, sample sizes within the BMC are too small to confirm this. Thus, future studies focussing on song and genetic divergence as well as song discrimination across the zone of secondary contact between *O. s. spaldingii* and *O. s. melasmenus* will clarify whether the results of this study are applicable across the species range, and whether *O. s. spaldingii* and *O. s. melasmenus* discriminate between each others' songs in the potential 'hybrid' zone.

CHAPTER 6   Discussion

6.1   Synthesis

The goals of this PhD were to determine the relative influence of different evolutionary processes in promoting geographic variation in song, morphology and molecular genetics in the Chowchilla, and to clarify the function and possible consequences of such variation. Specifically, this PhD aimed to (1) quantify the extent and pattern of large-scale geographic variation in song, as well as in morphology and neutral molecular genetic markers across the Chowchilla's range; (2) determine the evolutionary, cultural and social processes creating small-scale variation in song in order to clarify the functional significance of local song variation; and (3) determine whether birds discriminate between local and foreign song dialects to further establish the possible functions of geographic variation in song as well as its evolutionary consequences, i.e. reduced gene flow or reproductive isolation. Following is a summary of the main results of this project.

6.1.1 Large-scale song variation

Historically isolated populations could be clearly distinguished by their spectral song characteristics, particularly by bandwidth and peak frequency. I also found striking song divergence across the Black Mountain Corridor, a known historical climatic barrier. Northern refugia showed significantly narrower bandwidths and higher peak frequencies than southern refugia. Neither body size differences nor natural selection associated with differences in habitat structure appeared to have influenced patterns of song divergence in the Chowchilla. Body size generally affects peak and dominant song frequencies, neither of which differed significantly with body size in the Chowchilla. I also found no effect of broad habitat type on song divergence. There was no clustering of songs according to vegetation type in multidimensional space, and variation in song characteristic within vegetation types was as great as that among vegetation types. Therefore, given the known history of population isolation in Pleistocene refugia, patterns of large-scale song divergence in the Chowchilla were most likely influenced by vicariant isolation during the Pleistocene glaciations followed by cultural drift.

6.1.2 Small-scale song variation

Chowchillas showed exceptional small-scale spatial variation in their song, and song element similarity was significantly correlated with geographic distance. Song similarity decreased sharply at approximately one kilometre, confirming the existence of song neighbourhoods that share near identical songs. This pattern is consistent with the idea that Chowchillas learn their songs after dispersal when settling into a new group. In addition, neighbours shared significantly more syllables than non-neighbours. High levels of sharing among neighbours are thought to facilitate effective territory establishment, maintenance and defence. Therefore, given the aggressive nature of

Chowchilla vocal interactions, the pattern of high similarity among neighbours suggests that song has a strongly territorial function.

6.1.3 Molecular genetic divergence

Molecular genetic analyses clearly identified the presence of two distinct Chowchilla mtDNA control region lineages to the north and south of the BMC. Patterns of sequence divergence among Chowchilla populations south of the BMC suggest widespread recolonisation of this southern region from a single small refuge. This same recolonisation pattern was not as clearly evident among northern populations, possibly due to recolonisation from several refugial sources, or because genetic bottleneck effects that occurred north of the BMC were less severe than those experienced by southern populations. Sequences from the BMC itself fell within the southern haplotypes, suggesting that the BMC was recolonised from the south. These patterns are congruent with molecular divergence observed in several other Wet Tropics endemics and have been consistently interpreted as clear evidence of historical bottlenecks followed by drift in isolation and subsequent expansion to current distributions.

6.1.4 Morphological divergence

Chowchillas show striking and contrasting patterns of morphological divergence across the BMC depending on the character trait examined. Firstly, all northern birds were significantly smaller than southern birds despite consistent sexual size dimorphism in both northern and southern lineages. Secondly, independent of differences in body size between lineages or sexes, absolute bill length did not vary significantly among individuals throughout the chowchilla's entire range.

Molecular genetic divergence has been observed across the BMC in many Wet Tropics endemic taxa. However, no corresponding phenotypic divergence has previously been documented. This has led previous authors to the conclusion that genetic drift in isolation does not produce phenotypic divergence among populations in these systems because of the relative importance of other evolutionary processes such as stabilising selection. These data and conclusions stand in stark contrast to my own findings for Chowchilla. The strong morphological divergence observed in Chowchillas could potentially be explained by adaptation to divergent habitats. However, rainforest types inhabited by Chowchillas north and south of the BMC are no more distinctive or variable than within each of these regions. This observation combined with the lack of habitat effects on song divergence as well as the lack of variation in bill morphology make adaptive divergence linked to habitat characteristics unlikely. Therefore, in contrast to previous generalisations, data from my study suggest that genetic drift in isolation can indeed influence both morphological and/or behavioural traits significantly in some taxa.

Given the observed differences in body size, the lack of a corresponding (allometric) change in bill morphology must result from the action of an alternative evolutionary process, most likely strong stabilising selection associated with trophic constraints and/or a specialised feeding niche. Despite such strong divergence in many character traits, there was a distinct lack of variation in absolute bill size.

6.1.5 Sexual dimorphism

Chowchillas also showed consistent patterns of sexual dimorphism in both size and plumage colour both north and south of the BMC, despite absolute size differences among lineages. Sexual size dimorphism is commonly attributed to either differential niche utilisation by each sex, or intra-sexual competition. Based on the negligible variation in bill size between the sexes, as well as on field observations, male and female Chowchillas are thought to share the same feeding niche. This makes differential niche partitioning unlikely. Chowchillas are also very territorial, suggesting that male-male competition may play a role in creating size dimorphism. However, both sexes are involved in territorial defence. Social group living and reversed sexual colour dimorphism further blur the clear differences in sex roles needed to explain size dimorphism using standard theoretical models. Despite clear, conventional sexual size dimorphism, Chowchillas show reversed sexual plumage dimorphism with females sporting a bright orange chest. While the exact evolutionary processes responsible for sex-specific differences in the Chowchilla remain unclear, current theory suggests the consistent patterns of dimorphism observed among lineages must revolve around a complex mating system that involves elements of sexual selection and/or intra-sexual competition.

6.1.6 Geographic song discrimination

Chowchillas recognised and approached all song dialects tested, with an apparent lack of discrimination between subspecific dialects. However, Chowchillas engaged in territorial song quicker and more often upon hearing local song, with progressively fewer responses to increasingly distant dialects. Similarly, song latency also increased with increasingly distant dialects. These results confirm that the main function of local dialects in this species is efficient territory defence, which suggests that neighbours pose the greatest threat to a group's recourses. Surprisingly, Chowchillas sang more often in response to song from a different lineage than to equally distant, within-subspecific song. This shows that despite the strong divergence in song, morphology and genetics between the two lineages, Chowchillas still respond to song from the different subspecies. Therefore, song divergence is unlikely to create a pre-mating barrier to gene flow in this species.

6.2 Conclusions

Striking geographic variation in song as well as in body size across the Chowchilla's range mirrors patterns of molecular genetic divergence, i.e. distinct differences in both characteristics between northern and southern molecular lineages. Such congruence of divergence across multiple character

traits suggests that similar evolutionary processes are likely responsible, specifically genetic drift in isolated refugial populations. In general, the results of this study clearly show that different evolutionary processes can have significant and contrasting roles in creating and maintaining divergence, as measured by different character traits, and that studying these processes in isolation is unlikely to lead to a complete understanding of how species developed their current shape, form and behaviour.

6.3   Future Research

The findings of this PhD lead to very intriguing questions for future research. For example, little is known about the Chowchilla's social and mating system, which would help answering many questions arising from this thesis. In particular, how far do birds actually disperse? This question is linked with song learning and sharing patterns, relatedness within and between groups of Chowchillas, and the likelihood of birds migrating across the BMC and between refugia. In addition, knowledge of their mating system would also clarify the relative importance of sexual selection in shaping the unusual patterns of sexual size and plumage dimorphism.

More detailed research into the differences between habitats, available resources, inter- and intra-specific competition and foraging strategies either side of the BMC would also help distinguish between the specific evolutionary forces operating within the isolates. In particular, this approach would clarify the influence of natural selection on this system.

Molecular genetic studies using higher resolution techniques (such as microsatellites) would help determine whether the historical divergence found in Chowchilla mtDNA is still current, i.e. whether populations may still be isolated today. Such research would also help clarifying the mating system of the Chowchilla, how far they disperse, and whether dispersal is sex-biased.

Finally, given the fact that most previously studied taxa do not show any phenotypic divergence within this rainforest system, a thorough analysis of phenotypic variation in other Wet Tropics endemics would yield further insights into the relative importance of the different evolutionary processes in such a system.

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