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Phylogenetics and speciation of African *Bradypterus* and the *Apalis thoracica* complex

by

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For my mom

Thanks for your love, patience and making this possible

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'Science never pursues the illusory aim of making its answers final, or even probable. Its advance is, rather, towards the infinite yet attainable aim of ever discovering new, deeper and more general problems, and of subjecting its ever tentative answers to ever renewed and ever more rigorous tests'

- K.R. Popper (1959)

DECLARATION

I declare that the work presented in this thesis is my own, unaided work, except as acknowledged in the text. It is submitted as the requirement for the degree of Master of Science at the University of Pretoria, Pretoria, South Africa. It has not been submitted before for any degree or examination at this or any other university.

Signed on this 15th day of June 2003

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ABSTRACT

Phylogenetics and speciation of African *Bradypterus* and the *Apalis thoracica* complex

by

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Presented in this thesis is the mitochondrial DNA (mtDNA) cytochrome *b* gene analysis of the phylogenetic relationships within the *Apalis thoracica* complex (600bp) as well as among selected African *Bradypterus* species (603bp). Within *A. thoracica* 28 individuals from 20 subspecies were analysed. There was only molecular support for 13 historically isolated clades as opposed to the 21 subspecies recognized based on morphology. Among these molecularly defined clades were clades *lynesi*, *fuscigularis* and *flavigularis*, all three of which are of conservation concern and were previously treated as species by some based on their distinct morphology. Molecular clock dating estimated the clades to be of Pliocene age, whereas genetic differentiation within clades was of Plio-Pleistocene to Pleistocene age. This result is consistent with that of other African montane birds. Under the phylogenetic species concept all 11 clades could be recognised as species although a multifaceted approach to species recognition which will include vocalisation data is suggested. Contrary to expectation, the isolated East African clades did not exhibit more variation than the continuously distributed clades within southern Africa. In fact, significant phylogeographic breaks were identified within South Africa that need

further investigation. For the African *Bradypterus*, 13 individuals from six of the 10 African species were analysed. These species were not monophyletic. Based on sequence divergence *B. victorini* was as different from the other members of the genus as it was from the outgroups. In addition its song is very distinct and both males and females sing. Therefore, based on the molecular results presented in this thesis as well as vocalisation data and, it is proposed that *B. victorini* is not a *Bradypterus*. This result is particularly significant in light of the growing evidence that the Cape region acted as a repository for ancient animal taxa and holds implications for the conservation status of the region. The molecular data supported the classification of the taxa based on habitat and song but in addition clarified the placement of *B. victorini* and *B. sylvaticus*. The basal position of the latter among the remaining members of the genus indicates that *Bradypterus* is primarily a forest taxon that radiated into other habitats. It must however be kept in mind that the current dataset is based on six of the 10 African *Bradypterus* and none of the Asian species. The possibility of hybridisation between *B. sylvaticus* and *B. barratti* was raised and needs to be tested through additional sampling.

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CHAPTER 1

General Introduction

1.1 Background and broad overview

Africa's forests can be divided into two main groups: widespread lowland forest and montane forest restricted to higher peaks and rifts. Each forest type has its own characteristic suite of bird species. Because of their dependence on topography, the montane forests are more restricted in extent, and tend to occur in isolated pockets or clusters. Consequently, their bird populations are more fragmented, and often exhibit greater geographic variation than do lowland forest bird species. These montane forests are currently under increased pressure from human populations seeking fertile areas for agriculture. This presents a reason for concern since it is thought that much of the biological diversity of these areas remain hidden (Roy *et al.* 1998) and there is consequently a significant risk that genetically distinct forms will be lost simply through a lack of taxonomic recognition (Avise 1989).

Molecular techniques could aid in identifying hidden and cryptic diversity within montane forest taxa. In addition, when combined with analyses of phenotypic variation, reproductive isolation and evidence from geology and palaeoclimatology, molecular systematics can be very useful in the study of speciation (Templeton 1994; Moritz *et al.* 2000). The study of speciation aids our understanding of how historical processes shaped genetic and species diversity as well as how current evolutionary processes maintain phenotypic diversity (Moritz *et al.* 2000). This information is needed to devise conservation strategies (Rosenzweig 2001; Palumbi 2001; Western 2001) that are aimed at conserving both the irreplaceable genetic variation as well as phenotypic variation (Moritz *et al.* 2000). In this thesis the phylogenetic relationships within representative groups of birds with largely montane distributions, were used to assess the uniqueness of populations that inhabit different mountains and consequently also assess their conservation status. Ultimately the aim was to gain a better understanding of the region's biogeography and test models of speciation in montane forests by comparing phylogenetic patterns among similarly distributed montane biota.

1.1.1 The Afromontane region

The Afromontane region (Fig. 1.1) floristically consists exclusively of endemic or near endemic species (White 1981) and the region is sufficiently different from the surrounding lowlands such that it can be recognized as an archipelago, the Afro-montane Floristic Region (White 1978). This region essentially consists of three smaller regions (Fig. 1.1) that arose and were shaped independently from each other by different forces: the Cameroon highlands, Albertine Rift and eastern chain (including the Eastern Arch Mts. and Malawi Rift). Of these the eastern chain is the largest, most biologically unique and most diverse montane forest complex (Rodgers & Hamewood 1982; Stuart & Jensen 1985; Jensen & Brøgger-Jensen 1992; Lovett & Wasser 1993). Throughout most of the range the forests occur on the lower slopes of higher mountains and upper slopes of the lesser mountains (White 1981). To the south, however, especially in Pondoland and southern KwaZulu-Natal in South Africa, montane forest occurs at lower altitudes, with the forests reaching sea-level in the Western Cape (Cowling 1983; Vernon 1989).

1.1.2 Palaeogeographic and climatic influences

Palaeogeographic factors during the Tertiary period played an important role with regard to the formation of Africa's montane forests. A general drying in Africa from the beginning of the Tertiary, 65 million years ago (Ma) was one of the most important changes affecting the biota of montane regions. This spread of aridity was the result of the closing of the Tethys Sea during the Miocene and a consequent reduction in rainfall over the continent (Axelrod & Raven 1978). This resulted in a drastic fragmentation and contraction of the once extensive pan-African rainforest as well as the lowland equatorial rainforest that existed in the present day Sahara (during the Paleocene). The increase in aridity and the sinking of the Congo into a basin at the beginning of the Miocene/early Oligocene, in combination with rifting which raised the central plateau to its present level at the end of the Miocene, resulted in the formation of an "arid corridor" extending from NE Africa to SW Africa (Axelrod & Raven 1978; Lovett 1993). This corridor isolated eastern forests from the larger central and western forests (Axelrod & Raven 1978; Lovett 1993) and limited the migration of moist forest biota from the Guineo-Congolian rainforest in the west to the eastern lowland coastal forests (Lovett 1993). Finally, within the Plio-Pleistocene (1.8-2.0 Ma), the East African Rift Valley was formed which constitutes an important event that influenced montane biota. These changes undoubtedly greatly influenced speciation in forest-associated species.

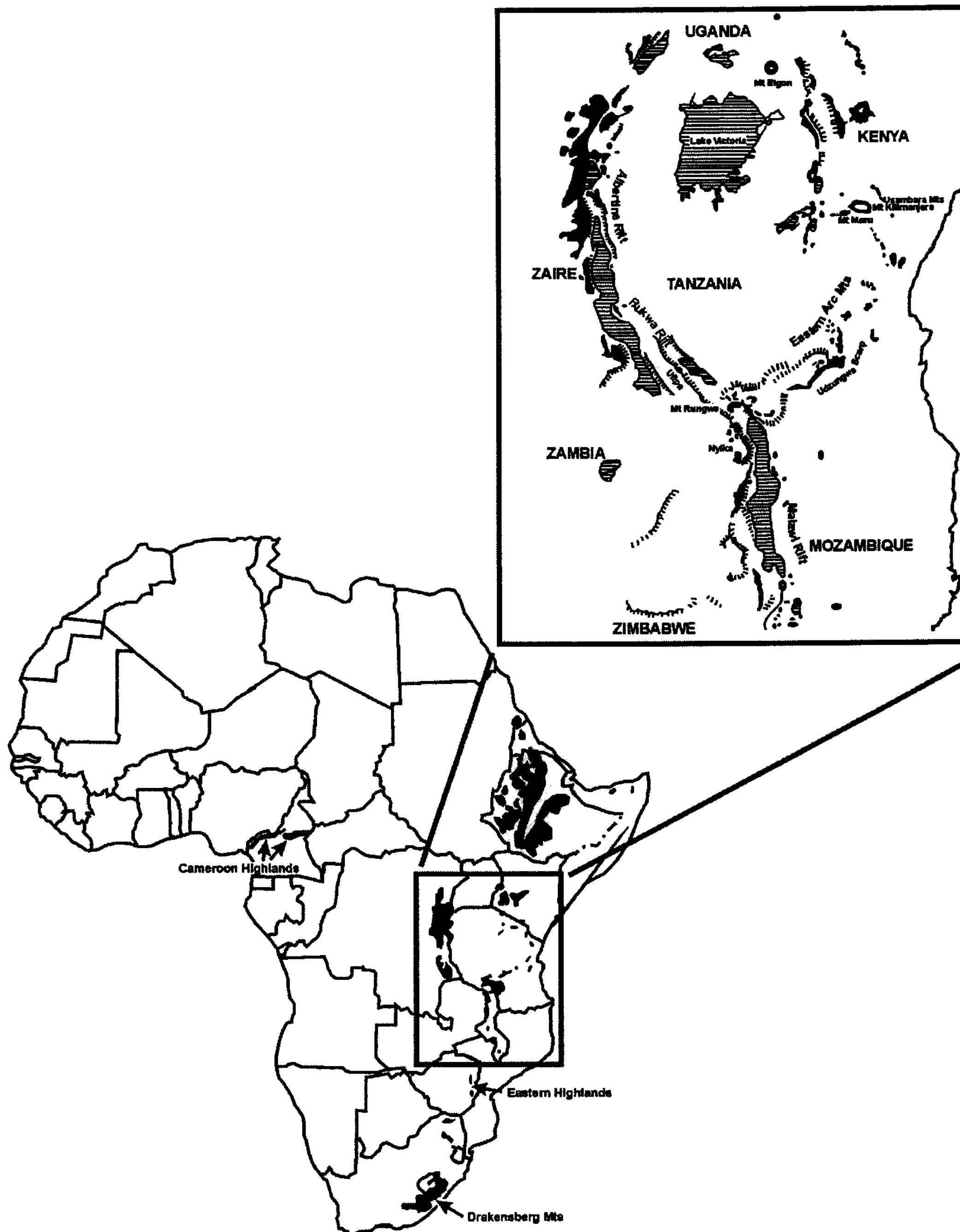

Figure 1.1 Geographic distribution of the African Afromontane archipelago. Shaded areas represent Afromontane forests, which occur at altitudes of 1500-1800m on most tropical mountains. The inset shows the location of the Eastern Arch Mts. and Albertine Rift as well as the Malawi Rift in more detail. South of the equator a decline in altitude occurs and montane forest is found a sea level at the southern tip of the continent (modified from White 1981). A montane avifauna containing 222 species is recognizable within this area (Dowsett 1986).

Milankovitch cycles were most pronounced during the Plio-Pleistocene (1.8 - 2.0 Ma), a period during which global cooling on 100000-year cycles gave rise to continental glaciers and was periodically interrupted by climatic warming cycles (Berger 1984; Bartlein & Prentice 1989; Dynesius & Jansson 2000) that lasted 10000 years. Much of the literature on biological diversification in the tropics has focussed on the possible effects of these Milankovitch cycles in combination with a general global cooling (Berger 1984; deMenocal 1995) on the size and location of the geographic distributions of species (Moreau 1966; Webb & Bartlein 1992; Dynesius & Jansson 2000).

1.1.3 Diversification of the montane avifauna

The origins of the disjunct distributions of montane forest birds have intrigued scientists for many years (Moreau 1966; Haffer 1969; Diamond & Hamilton 1980; Crowe & Crowe 1982; Roy 1997; Roy *et al.* 1998; Bowie *et al.* in review). At first it was thought that these disjunct patterns could only be explained by former forest connections between mountain blocks that are currently isolated and that climatic changes allowed extensive spread of montane forest, thus connecting areas that are now separated by lowland forest (Chapin 1923). In the "Pluvial theory" Moreau (1966) postulated that the spread in montane forests occurred during the glacial periods when lower temperatures resulted in a reduction of evaporation and hence an increase in the effectiveness of rainfall. This was argued to have resulted in the spread of Afromontane forests to lower elevations, bringing these forests in closer proximity and allowing continuous or almost continuous communication among montane species during glacial periods. During the interglacials, the montane forests once again contracted, retreating to higher altitudes and effectively isolating their avifaunas.

Today it is however more generally believed that glacial cycles in Africa were dry (as opposed to wet) and interglacials more humid (Livingstone 1975). It is hypothesized that speciation in the tropical forest biota was mediated by the formation of isolated forest refugia during dry periods (glacials of the Pleistocene) when forest cover contracted (Haffer 1974, 1982; Diamond & Hamilton 1980; Kingdon 1990). This Forest Refuge Hypothesis was developed by Haffer (1969) for avian speciation in tropical South America and has been subsequently applied to African forest avifauna by various authors (Diamond & Hamilton 1980; Crowe & Crowe 1982; Mayr & O'Hara 1986; Kingdon 1990; Fjeldså 1994; Fjeldså & Lovett 1997; Roy 1997). The hypothesis has however been criticised extensively (Endler 1982a, 1982b; Witmore & Prance 1987; Lynch 1988; Bermingham *et al.* 1992; Flenley 1993; Klicka & Zink 1997).

It has been assumed that speciation by isolation in forest refugia had its greatest effect during the Pleistocene when the Milankovitch cycles were most pronounced. Some authors have even proposed late Pleistocene origins for many taxa (Klicka & Zink 1997), but it has become clear that most avian species radiations predate the Pleistocene (Roy 1997; Avise & Walker 1998; Roy *et al.* 1998; Klicka & Zink 1999).

Furthermore it was also widely assumed that speciation by isolation in forest refugia had its greatest effect in the species-rich lowland regions (Haffer 1969, 1974; Diamond & Hamilton 1980; Crowe & Crowe 1982; Prance 1982; Mayr & O'Hara 1986; Witmore & Prance 1987). Molecular evidence, however, now suggests that areas of recent intensive speciation are located in montane habitats and date to the Plio-Pleistocene, whereas lowland forests contain mostly old species of pre-Pleistocene origin (Roy 1997; Roy *et al.* 1998). This led Roy (1997) to hypothesise that intensive speciation occurred in montane forests, with some of these novel species subsequently extending their ranges to lowland forests, which acted as sinks where taxa of pre-Pleistocene age collected.

The suggestion that there may have been considerable spread of forests in Africa after 18000 years ago (Livingstone 1975; Hamilton 1981) led Diamond & Hamilton (1980) to conclude that disjunctly distributed montane bird species have probably either flown from one area to the other or else once occurred in the intervening lowland forest. This would mean that birds were either very mobile or previously showed a much wider tolerance of altitudinal range. Existing evidence, however, suggests the opposite, that most forest birds are essentially sedentary, with very limited capacity to cross even small stretches of open habitat (Dowsett 1980a, b). It also seems unlikely that forest species that are now strictly high-altitude birds would have occurred in lowland forest in the past.

White (1981) commented that simple historical explanations could not adequately explain the disjunct distributions and diversity of relationships in the Afromontane region and that existing hypotheses would have to be modified with the discovery of new facts. Even today the various hypotheses (e.g. Moreau 1966; Haffer 1969; Diamond & Hamilton 1980; Crowe & Crowe 1982) remain largely untested or are not supported by the limited recent evidence (e.g. Roy 1997; Roy *et al.* 1998, 2001; Bowie *et al.* in press).

1.1.4 Vicariance or dispersal?

Prior to 1970, dispersal was regarded as the primary process responsible for the spatial and temporal patterns of biotic diversity (Zink *et al.* 2000). It was assumed that ancestral population dispersed across pre-existing barriers and that the new populations became isolated and evolved into new species (Udvardy 1969). A few years later the concept of vicariance was introduced (Nelson & Platnick 1981). Vicariance is geographic isolation, the division of an interbreeding population by the rise of a physical, isolating barrier such as a mountain chain (Croizat *et al.* 1974; Nelson & Platnick 1981; Nelson &Rosen 1981; Humphries & Pareni 1986; Myers & Giller 1988).

Proponents of vicariance speciation advocate that a rare but extensive dispersal event (range expansion) is followed by a series of allopatric isolation events (Bush 1975) and that these may be interrupted by occasional random dispersal events. If the isolation events affect many organisms simultaneously, congruent tree topologies will indicate that they are vicars. On the other hand proponents of the dispersal paradigm consider dispersal to have a much larger and more active role (Zink *et al.* 2000).

Both paradigms are compatible with allopatric speciation (Ebach & Humpries 2002), the most widely recognised mode of speciation (Chesser & Zink 1994). They do, however, differ with respect to the role that dispersal plays in allopatry. Although vicariance and dispersal were regarded as two opposing hypotheses (Brown & Lomolino 1998) some treat them as reconcilable (Craw *et al.* 1999) by applying a biogeographic model that involves alternating cycles of dispersal and vicariance (Crisci 2001). Comparisons of multiple phylogenetic hypotheses among similarly distributed montane biota could elucidate vicariance and dispersal events.

1.2 The study animals

Most taxa within the *Apalis thoracica* complex and most African *Bradypterus* warblers are associated with Afromontane forests and the adjacent scrub. In addition they have overlapping distributions in Africa (Fig. 2.1 & 3.1), which could allow the inference of common evolutionary processes and biogeographic hypotheses concerning the influence of paleogeographic and climatic changes on speciation in these forests.

1.2.1 *Apalis thoracica* complex

The Bar-throated *Apalis* (*Apalis thoracica*) complex is a striking example of geographic variation. Three of the 21 recognized subspecies (Urban *et al.* 1997) are of conservation concern including *A. [t.] lynesi*, *A. [t.] flavigularis* and *A. [t.] fuscigularis*. These are regarded as Vulnerable, Endangered and Critically Endangered, respectively (BirdLife International 2000) and although they are currently described as subspecies of *A. thoracica* (Urban *et al.* 1997) some (BirdLife International 2000) treats them as species.

The East African subspecies occur as a series of fragmented populations restricted to the higher mountains of East Africa (Fig. 2.1). Irwin (1966) has argued that there can be little if any gene flow between these populations under the present climatic conditions. Some of these East African populations are very distinct in plumage. South of the Zambezi River *A. thoracica* becomes less habitat specific, occurring in coastal scrub, riparian thickets and dense woodland as well as forest. The southern African subspecies (Fig. 2.1) occur contiguously from the southwestern Cape (South Africa) along the narrow coastal margin up to eastern Botswana and Zimbabwe (Harrison *et al.* 1997). Here there is much greater likelihood of gene flow between subspecies, and these taxa exhibit broad clinal trends in plumage colour. However, there is no evidence of regular movement in this species (Harrison et al. 1997), and none of 62 Bar-throated Apalises recovered subsequent to banding had moved at all (SAFRING unpubl. data).

1.2.2 African *Bradypterus*

The genus *Bradypterus* includes 18 species, 10 of which are African. Three species are southern African endemics: the Knysna Warbler (*Bradypterus sylvaticus*), Barratt's Warbler (*B. barratti*) and Victorin's Warbler (*B. victorini*). In addition, three of the African species are of conservation concern: the Knysna Warbler and Dja River Warbler (*B. grandis*) are listed as Vulnerable and Grauer's Scrub-Warbler (*B. graueri*) as Endangered (BirdLife International 2000; Barnes 2000). All the African *Bradypterus* species have fragmented or disjunct distributions with the exception of the Little Rush Warbler (*B. baboecala*), which occurs in reedbeds and is widespread (Fig. 3.1). The ephemeral nature of most wetland habitats, coupled with the widespread distribution of Little Rush Warblers, suggests that this species is more vagile than most *Bradypterus*, and this is supported by ringing recoveries, with one bird moving 19 km out of a sample of 55 recoveries (SAFRING unpubl. data).

Bradypterus is characterised by very uniform morphology, which renders morphological characters of little value in resolving species and racial affiliations and status. In addition, all African *Bradypterus* warblers are very secretive, which makes them difficult to observe and identify. Consequently, there has been a wide diversity of treatment in species limits and racial affiliations within *Bradypterus*. Their well-developed songs are highly species-specific and classifications based partly on habitat but mostly on vocalisation (Dowsett & Forbes-Watson 1993; Urban *et al.* 1997) have been used to infer relationships. The phylogenetic relationships of especially *B. victorini* and *B. sylvaticus* however remain uncertain.

1.3 Molecular markers

In the past, phylogenetic reconstruction, including those of *A. thoracica* and *Bradypterus*, was based almost exclusively on morphological characters as well as vocalisation in the case of *Bradypterus*. With the introduction of DNA technology, a wealth of new characters for systematic analysis became available and more emphasis was placed on estimating molecular phylogenies and using these to make inferences about evolutionary relationships (Moritz & Hillis 1996). Molecular phylogenies have since proven very useful in resolving species limits and affiliations, as well as in the identification of cryptic species (Irwin *et al.*2001a; Zimmer *et al.* 2001), especially in taxa where more traditional morphological methods have been inadequate or misleading guides to phylogenetic distinctions at the subspecies or species level (Avise 1989).

Incongruence between phylogenies derived from morphological versus genetic traits has been documented in other warblers (Hedges & Sibley 1994; Leisler *et al.* 1997) and is attributed to convergent evolution and specialising adaptations. Since the marker genes used for sequencing are usually not those controlling the development of the morphology of a species they are less subject to these problems (Avise 1994, Hillis *et al.* 1996).

1.3.1 Mitochondrial DNA

The characteristics of mitochondrial DNA (mtDNA) make this molecule extremely useful for constructing genealogies (Avise *et al.* 1979, 1987; Moritz *et al.* 1987; Harrison 1989). Mitochondrial DNA has a high substitution rate (Brown *et al.* 1979, 1982), which renders it useful in studies of recently evolved taxa. It also has a highly conserved gene order (Brown 1983), allowing the design of polymerase chain reaction (PCR) primers that can be used across a broad range of taxa (Kocher *et al.* 1989). It is thought not to be subject to recombination

(Arctander 1999; Merriweather & Kaestle 1999) a process that would erase the trace of lineages. There is however some evidence to the contrary (Eyre-Walker *et al.* 1999; Hagelberg *et al.* 1999; Bromham *et al.* 2003). The molecule is maternally inherited and effectively haploid in transmission across generations, which reduces the effective population size when compared to nuclear genes (Avise *et al.* 1987; Harrison 1989). In addition the wealth of literature on animal and avian mtDNA allow comparisons between studies (Mindell 1997 and references therein). Lineage sorting may result in disagreement between gene trees and species trees (Neigel & Avise 1986; Hoelzer & Melnick 1994), but under certain conditions, as discussed by Hoelzer (1997) and Moore (1997), it is more likely for the mitochondrial-haplotype tree to agree with the species tree than the nuclear-gene tree (Moore 1995).

Due to the advantages of using mtDNA it has become a popular source of DNA sequencing data for phylogenetic analysis. It has proven a useful marker for elucidating phylogenetic relationships at different taxonomic levels (Avise 1994) and in studies of species boundaries (Wayne 1992). More applicable to the present study, is the fact that it has been used to evaluate the taxonomic status of avian plumage variants (Avise & Nelson 1989). Wiens & Penkrot (2002) have argued that mtDNA data may have important and previously unappreciated advantages for species delimitation.

1.3.2 General considerations regarding methods of inferring molecular phylogeny

There are three widely used methods of molecular phylogenetic inference: neighbour-joining, maximum parsimony and maximum-likelihood. All three methods have advantages and drawbacks (Siddall 1998 and references therein), but by comparing the results of all three most of their shortcomings can be compensated for.

The distance-based method, neighbour-joining (Saitou & Nei 1987), is a quick method of creating a tree topology based on the distances among sequences and allows the estimation of sequence divergences that can be compared among species, genera, etc. These divergences can be used to apply a molecular clock to the data (Page & Holmes 1998). However, it does not discriminate between similarity as a result of common decent or similarity due to convergence. Furthermore, being a phenetic method, it doesn't reflect ancestry, rather only how similar sequences are (Page & Holmes 1998).

On the other hand the maximum parsimony method (Kluge & Farris 1969; Farris *et al.* 1970) is a discrete method that uses sequences rather than pairwise distances to choose a tree (or trees) that requires the fewest evolutionary changes. It is a character-based method that uses synapomorphies to infer relationships (Page & Holmes 1998). Unfortunately it is subject to long-branch attraction (Hillis *et al.* 1994) when rates of evolution vary considerably among sequences or when the data contain many divergent characters. The maximum-likelihood model (Felsenstein 1973, 1981) is not as sensitive to long-branch attraction but could become prone to long-branch repulsion especially when dealing with closely related taxa that have long branches (Siddall 1998). This is the only method that follows a model-based approach, that estimates the tree topology most probable under the set model of evolution, i.e. it chooses the tree (or trees) that of all possible trees is the most likely to have produced the observed data. Furthermore it calculates the likelihood that a particular tree generated is the best fit to the data provided (Page & Holmes 1998). This method is however very time consuming and the fact that it requires a model of evolution is both a strength and a weakness (Page & Holmes 1998).

Phylogenetic inference using mtDNA may become obscured by saturation (Meyer 1994; Krajewski & King 1995). This occurs when the number of transitions and transversions in pairwise comparisons of diverging taxa increase linearly over time up to a point when transitions level off because of multiple substitutions (Moore & DeFilippis 1997). This is expected to occur more rapidly in the third codon position because changes at this position (particularly transitions) are degenerate and thus occur more frequently than changes at first or second positions. As a consequence, saturation may lead to an underestimation of the number of mutations (transitions and transversions), an underestimation of the actual amount of evolutionary change as well as weak phylogenetic signal (Moore & DeFilippis 1997; Page & Holmes 1998) and therefore needs to be compensated for (Page & Holmes 1998).

1.3.4 Molecular clock

In addition to providing clues toward elucidating the causes of speciation, trees derived from DNA sequences contain information on the relative timing of reconstructed speciation events (Hey 1992; Purvis 1996; Barraclough & Nee 2001). This information can be extracted by applying a molecular clock to the data. The molecular clock concept is based on the assumption that if differences in DNA sequences accumulate at roughly a constant rate over time (Zuckerkandl & Pauling 1965; Brown *et al.* 1979) the levels of DNA sequence divergence can be used to date splitting events.

In reality however, it has become clear that the application of a molecular clock is controversial and is complicated by: variation in mutation rate and substitution rate between lineages (Li 1993a,b; Hillis & Moritz 1990), the influence of life history characteristics such as body size, metabolic rate and generation time (Martin & Palumbi 1993) as well as a poor fossil record for some biota such as passerines (Van Tuinen & Hedges 2001). It has also been found that molecular data consistently yield deeper dates than indicated by stratigraphic dates (Hedges *et al.* 1996; Hedges & Kumar 2003) and that there is a methodological bias towards overestimation of times of divergence (Rodriguez-Trelles *et al.* 2002). Numerous authors have proposed tests to evaluate whether a data set is consistent with a null hypothesis of a molecular clock (Felsenstein 1981; Templeton 1983; Muse & Weir 1992; Tajima 1993). Various methods of estimating divergence times in the absence of a molecular clock have also been proposed (Sanderson 1997; Thorne *et al* 1998; Huelsenbeck *et al.* 2000; Yoder & Yang 2000).

Despite the inability of the molecular clock hypothesis to perfectly and accurately explain the substitution process (Brown *et al.* 1979; Shields & Wilson 1987), it remains a powerful way of crudely estimating the timing of divergence events in evolutionary biology (Beerli *et al.* 1996, Sanderson 1998). It provides a timescale for evolution (Huelsenbeck *et al.* 2000) and in doing so allows one to determine the geological and ecological context of divergence events (Yoder & Yang 2000). This facilitates evaluation of competing biogeographic hypotheses (Zink & Slowinski 1995; Hillis *et al.* 1996; Klicka & Zink 1997; Arbogast & Slowinski 1998; Gibbons 1998) and ultimately contributes to a better understanding of speciation and dispersal mechanisms (Yoder & Yang 2000).

1.4 Phylogeography

Phylogenetic trees contain an indirect record of the speciation events that have led to present-day species (Barraclough & Nee 2001) and together with information on the geographic and ecological attributes of species they can provide clues on the mechanisms of speciation (Hey 1992; Barraclough *et al.* 1998; Barraclough & Nee 2001). The field of phylogeography does exactly this. It simultaneously analyses spatial and temporal patterns by combining information on gene trees with geographic distributions and thereby makes conclusions about the processes and principles driving the geographic distribution of lineages (Avise *et al.* 1987; Avise 1998, 2000). By applying methods such as likelihood, coalescence (Kuhner *et al.* 1995, Beerli & Felsenstein 1999) and nested clade analysis (Templeton 1998) phylogeographic patterns can be interpreted in the light of evolutionary and biogeographic models (Arbogast & Kenagy 2001).

The influences of historical factors including dispersal and vicariance can then be investigated. Furthermore, population genetic parameters such as within-population heterozygosity, between-population gene flow as well as the genetic distinctiveness of taxonomic units can be determined.

Mitochondrial DNA has been the workhorse of phylogeography and most studies have dealt with the historical, phylogenetic components of the spatial distribution of mitochondrial gene lineages (Avise 2000; Hare 2001). However, because mtDNA is maternally inherited, the information it contains is restricted to that part of the genome. There is therefore a need for a general analytical approach that can be applied across independent loci (Hare 2001), and the future value of nuclear DNA in this respect has been emphasised by Avise (1998). The study of phylogeography elucidates the evolutionary history of species and can identify historically isolated lineages that need to be conserved, these inferences are needed in both *A. thoracica* and the African *Bradypterus* warblers.

1.5 Comparative phylogeography

An extension of the phylogeographic approach is a comparison of phylogeographic or geographic patterns of evolutionary subdivision patterns across multiple co-distributed taxonomic groups, termed comparative phylogeography (Cracraft 1989; Zink 1996; Avise 2000). It aims to identify suites of species that have a common history of vicariance (Moritz & Faith 1998; Bermingham & Moritz 1998). Consequently this field can contribute to the understanding of historical responses to changes in the landscape (Moritz & Faith 1998). It has led to the discovery of previously unrecognised biogeographic patterns which suggest that vicariance has played a more important role in the historical development of modern biotic assemblages than previously thought (Arbogast & Kenagy 2001). Comparative phylogeography allows the identification of evolutionary isolated areas, which can inform conservation strategies (Moritz & Faith 1998; for an application in birds see Smith *et al.* 2000).

1.6 Species Concepts

The interpretation of biogeographic hypotheses on diversification or speciation will be influenced by the definition of a species. Over the last couple of decades there has been much debate on species concepts (e.g. Coyne *et al.* 1988; Cracraft 1989; Hull 1997; Wheeler & Meier 2000 and references therein). The prevailing species concepts include the Biological (Dobzhansky 1940; Mayr 1970), Recognition (Paterson 1985), Phylogenetic (Cracraft 1989) and Cohesion

(Templeton 1989) Species Concepts, all of which have received support as well as criticism (Cracraft 1983; Amadon & Short 1992; Zink & McKitrick 1995; Haffer 1997; Johnson *et al.* 1999 and references therein). It is not the purpose of this thesis to deal with all of these (for a review of species concepts see Cracraft 1983, 1989, 1997; Amadon & Short 1992; Haffer 1992, 1997; Zink & McKitrick 1995; Corbet 1997; Hull 1997; Zink 1997). In this thesis the phylogenetic species concept as well as a 'Multifaceted Approach' (Crowe *et al.* 1994; Crowe 1999) to species recognition was applied. The former is defined as "An irreducible (basal) cluster of organisms, diagnosably distinct from other such clusters, and within which there is a parental pattern of ancestry and decent" (Cracraft 1989). The latter advocates the delineation of bird species based on congruent patterns in as many sets of characters as possible e.g. song, morphology and genetics (Crowe *et al.* 1994; Crowe 1999).

1.7 Thesis Preview

The aim of this project was to resolve issues relating to phylogenetic relationships (phylogenetics) and speciation in African *Bradypterus* Warblers and the *Apalis thoracica* complex. In addition, both contain taxa that are of conservation concern and it was hoped that DNA analysis would shed light on the phylogenetic relationships and phylogenetic distinctiveness of these taxa, which would have an impact on their conservation status. Geographic patterns of mtDNA and morphological variation are relevant to the study of speciation (Moritz *et al.* 2000). Similarly mitochondrial phylogeographic patterns and estimations of times of divergence were used here to formulate biogeographic hypotheses on diversification in these two groups. Due to the small number of *Bradypterus* samples and the lack of geographic representativeness of these samples, however no phylogeographic hypothesis could be tested for this genus and the focus of the chapter was shifted to the uniqueness of Victorin's Warbler.

1.7.1 Chapter 2

Chapter 2 discusses the molecular phylogeny and speciation in the *A. thoracica* complex. Urban *et al.* (1997) treats the races as incipient species. In addition, three of the races are treated as species by BirdLife International (2000).

The aims of this study were as follow:

(i) to use mtDNA to study the genetic diversity among the subspecies and identify historically isolated lineages

(ii) to compare molecularly defined lineages to the morphologically defined races

(iii) to compare genetic diversity and the level of molecular differentiation among contiguously distributed races with that among isolated races

(iv) to identify vicariant patterns with co-distributed montane avifauna / compare phylogeographic patterns within *A. thoracica* with patterns in other co-distributed montane avifauna in order to elucidate vicariant patterns

(v) to roughly estimate times of divergence by applying a molecular clock

(vi) and to formulate a possible biogeographic hypothesis on diversification in the complex.

1.7.2 Chapter 3

This chapter deals with the phylogenetic relationships among and speciation in six of the 10 African *Bradypterus* warblers for which there has been wide diversity of treatment in species limits and racial affiliations.

The aims of this chapter were:

(i) to assess the phylogenetic relationships among six of the 10 African *Bradypterus* Warblers

(ii) to compare the resulting mtDNA phylogeny with the current classification based on a combination of habitat choice and additional analyses on vocalisation

(iii) to roughly estimate times of divergence by applying a molecular clock.

1.7.3 Chapter 4

Finally, chapter 4 presents an integrated summary of the main findings of the thesis.

CHAPTER 2

Molecular phylogeny of the *Apalis thoracica* complex

Abstract

The Bar-throated Apalis, *Apalis thoracica* is a polytypic warbler confined to forests and thicket vegetation in southern and eastern Africa. Historically it has been split into a number of species, largely due to marked plumage differences among isolated populations found in mountain forests of eastern Africa. All are however currently treated as subspecies with the exception of races *lynesi*, *fuscigularis* and *flavigularis* which are of conservation concern and have been listed as species in the latest red data book for birds. We inferred the relationships among 19 of the 21 recognised subspecies from analysis of 600 base pairs (bp) of the mitochondrial DNA (mtDNA) cytochrome *b* gene. Phylogenetic analysis identified 11 strongly differentiated mtDNA clades within the monophyletic complex. The levels of sequence divergence among the 11 proposed clades exceeded values typically found between subspecies and were in the range of species. The molecular data indicated that not only are the threatened taxa historically isolated clades, all the identified clades were distinct on a molecular level. Therefore, under a multifaceted approach to species recognition, additional data (vocalisation and morphology) are needed to establish the .conservation status of all the clades and formulate conservation policies to protect them. The deep molecular divergences within the contiguously distributed clades in South Africa were not reflected in plumage differences and presents previously overlooked biodiversity within South Africa. Application of a molecular clock of 2% sequence divergence per years (Myr.) suggested that the earliest divergences within *A. thoracica* occurred during the Pliocene, with the remainder dating to the Plio-Pleistocene and Pleistocene. This, together with the basal grouping of the East African clades, suggests that *A. thoracica* originated in East Africa, then expanded southward, colonising suitable habitats without gene flow in the opposite direction. There might also have been subsequent southward colonisations that did not reach the most southern clades. The timing of speciation in East Africa is consistent with the radiation of other montane birds, including greenbuls, thrushes and sunbirds.

Introduction

The montane component of African forests is characterised by high levels of regional endemism such as the Adamawa highlands of south-west Cameroon and the Albertine Rift of the eastern Democratic Republic of the Congo. The highlands of south-eastern Africa are the largest and most diverse montane forest complex (Rodgers & Hamewood 1982; Stuart & Jensen 1985; Lovett 1988; Jensen & Brøgger-Jensen 1992; Lovett & Wasser 1993), extending along the eastern branch of the Rift Valley from Kenya to Malawi, and then south to the eastern highlands of Zimbabwe and the escarpment of South Africa (White 1981; Cowling 1983; Vernon 1989). Many forest-associated avifauna, for example the Yellowthroated Warbler, *Phylloscopus ruficapillus*; the Cape Robin, *Cossypha caffra* and the Cinnamon Dove, *Aplopelia larvata*, follow the distribution of the Afromontane forests (Urban *et al.* 1997) but little is known about the evolution of these forest birds (e.g. Roy 1997; Roy *et al.* 1997; Roy *et al.* 1998, 2001). Several early hypotheses (e.g. Moreau 1966; Haffer 1969; Diamond & Hamilton 1980; Crowe & Crowe 1982) have tried to explain the origin of the disjunctions seen in many of the Afromontane avifauna but remain largely untested or are not supported by the limited recent evidence (e.g. Roy 1997; Roy *et al.* 1997, 1998; 2001; Bowie *et al.* in review). The numerous forest fragments result in local patterns of endemism, but many species occur broadly through all or part of this Afromontane forest complex, allowing the inference of common evolutionary processes through comparative phylogeographic studies.

One such species is *Apalis thoracica*, a small, insectivorous warbler that exhibits striking phenotypic geographic variation (Urban *et al.* 1997) throughout its distribution in the Afromontane archipelago (Fig. 2.1). The head plumage exhibits different combinations of brown, grey or sometimes black, whereas the back is either grey or green and the under-parts white or yellow (Urban *et al.* 1997). All races have a black band separating the pale throat from the breast except in *fuscigularis* and *lynesi* where the entire throat is black (Urban *et al.* 1997). These two subspecies are confined to single mountain peaks in eastern Africa. Other East African taxa, *pareensis*, *uluguru*, *flavigularis* and *whitei*, also have disjunct distributions, restricted to the higher mountains above 1300 m and are rather distinct in plumage, suggesting that there is little if any gene flow between them under current climatic conditions (Irwin 1966). By comparison, the southern African subspecies are less variable in plumage (Lawson 1965; Clancey 1980; Urban *et al.* 1997) and from Zambia and Malawi southwards variation in plumage is broadly clinal (Lawson 1965). This clinal variation makes it very difficult to distinguish between neighbouring

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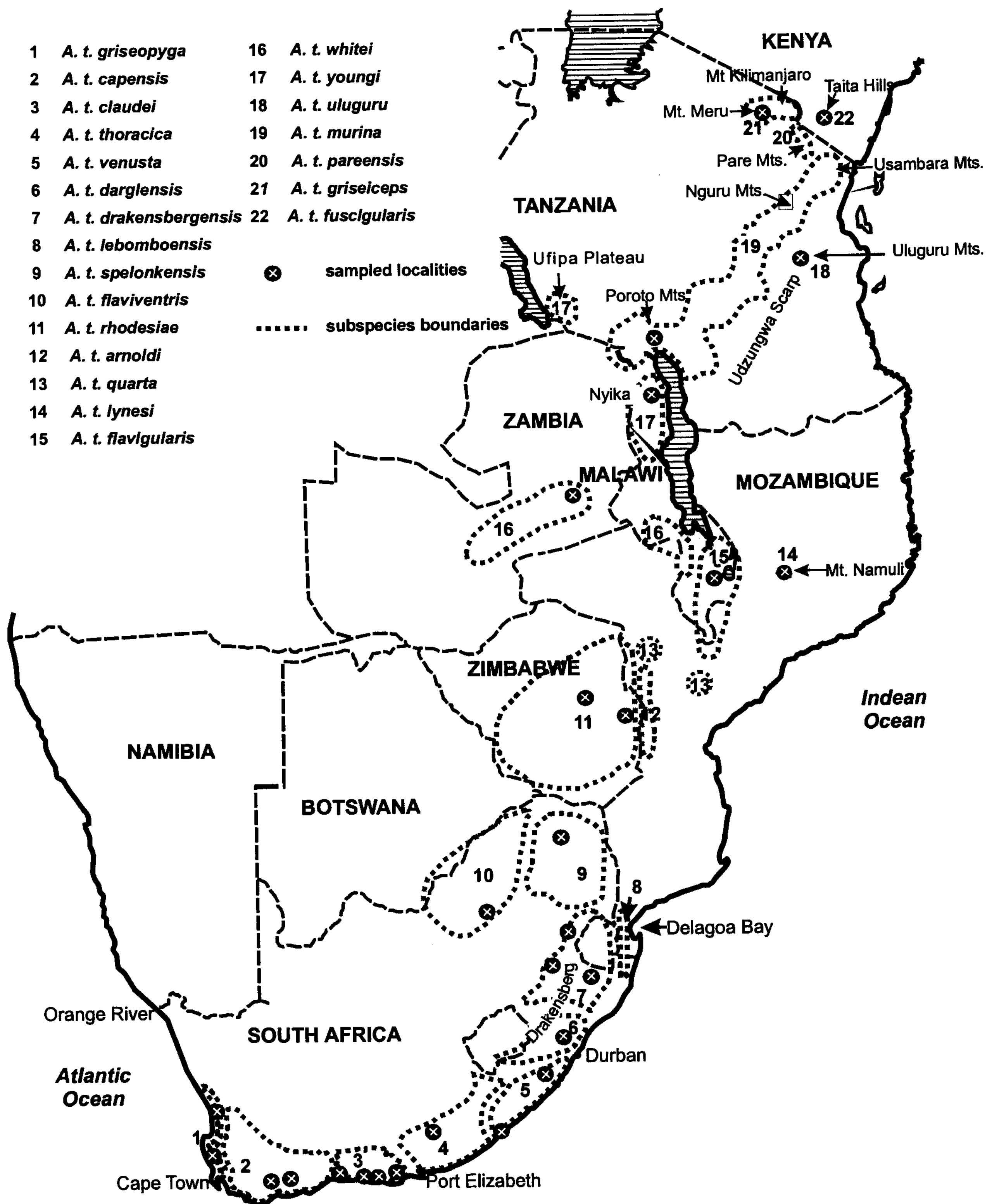

Figure 2.1 Map of southern and East Africa showing the geographic distribution and sampling localities for the races of *Apalis thoracica*. Numbers represent subspecies as explained in the key and some of the relevant mountain ranges are indicated. This map is a modification of Clancey (1980) and Urban *et al.* (1997).

subspecies, especially within South Africa where most subspecies have contiguous ranges (Fig. 2.1). The more extensive range in the south of its distribution results from a broader range of habitats occupied, including not only Afromontane forest, but also adjacent savannah, woodland and coastal dune scrub and there is a possibility of gene flow between neighbouring subspecies.

Phylogenetic relationships among the subspecies are unclear. The pattern of geographic variation within *A. thoracica* has been suggested to represent incipient speciation (Urban *et al.* 1997). Subspecies *fuscigularis* (Taita Hills, Kenya) and *lynesi* (Mount Namuli, Mozambique), were listed as incipient species that are at risk or requiring monitoring in Africa by Collar & Stuart (1985) and were treated as distinct species by BirdLife International (2000), as was *flavigularis* (southern Malawi). Several other subspecies were also previously treated as distinct species. Better understanding of the phylogeny of the *A. thoracica* complex thus has direct conservation relevance, and would also provide insight into speciation processes. The validity of the assumption that subspecies can be used as indicators of evolutionary processes depends on the criteria used to subdivide a species into subspecies (Oatley 1989; Patten & Unitt 2002). The characters used to define taxa are therefore very important and it has been noted that problems of character adaptation and convergence may sometimes obscure morphological analysis, as seen in other avifauna such as sylviid warblers (Leisler *et al.* 1997). Molecular data are less prone to these two problems (Hillis & Moritz 1990) and therefore molecularly defined clades are in such instances more useful for reconstructing/inferring evolutionary histories. The application of the molecular clock to the branching points of a phylogeny, although criticised (Hillis & Moritz 1990; Li 1993a,b; Hedges *et al.* 1996; Rodriguez-Trelles *et al.* 2002), allows a rough estimate of time of divergence (Brown *et al.* 1979; Shields & Wilson 1987) and can be used in formulating preliminary biogeographic hypotheses (Bermingham & Avise 1986). Clarification of the evolutionary relationships and history within this complex could add to our understanding of the regional biogeography of the Afromontane archipelago.

Mitochondrial DNA has proven to be a useful molecular marker in evaluating the taxonomic status of avian plumage variants (Avise & Nelson 1989) as well as studies of species boundaries (e.g. Avise *et al.* 1987; Shields & Helm-Bychowski 1988; Avise & Nelson 1989; Harrison 1989; Zink & Blackwell 1998). The mtDNA cytochrome *b* gene in particular has often been employed for studies on bird phylogeny (e.g. Birt-Friesen *et al.* 1992; Helm-Bychowsky & Cracraft 1993; Hedges & Sibley 1994; Helbig *et al.* 1995; Mindel 1997 and references therein; Helbig & Seibold 1999; Lougheed *et al.* 2000; Johnson 2001; Klicka *et al.* 2001; Roy *et al.* 2001; Burns *et al.* 2002; Salzburger *et al.* 2002; Klicka *et al.* 2003; Packert *et al.* 2003). Although single gene histories

alone are insufficient evidence for species diagnosis (Sites & Crandall 1997), mitochondrial gene trees and mitochondrial divergences allow an indirect estimate of historical isolation and allows genealogical traces to be followed across genetic boundaries between populations, species and higher taxonomic levels (Bermingham & Moritz 1998).

In this study I present a molecular phylogeny of the Bar-throated Apalis, which is based on sequences from the mitochondrial cytochrome *b* gene. The mtDNA sequence data were used to study the genetic diversity among the subspecies and identify historically isolated clades, which were then compared to morphologically defined races (Urban *et al.* 1997). In particular it was tested whether the morphologically distinct subspecies *lynesi*, *fuscigularis* and *flavigularis* are distinct genetically. Since patterns of diversification are usually more marked in taxa where there is some barrier to gene flow (Avise *et al.* 1987), the isolated East African subspecies were expected to exhibit more variation than the continuously distributed subspecies of southern Africa. It was also investigated whether the timing of the radiation within this complex was consistent with that of other montane birds.

Materials and methods

Samples

Blood or tissue samples were obtained from birds caught in mist-nets or collected under permit. Blood was taken from the brachial vein and stored in blood storage buffer (0.1M Tris-HCL, 0.5M EDTA·Na_2, 1.0M NaCl, 0.5% SDS) with a 1:10 ratio of blood to buffer. Heart, liver or pectoral muscle tissue was stored in DMSO/NaCl (Amos & Hoelzel 1991), a mixture of 100% EtOH and formalin or kept frozen at -80°C. Fresh tissue samples were obtained from all taxa, but only foot scrapings were available for *pareensis* (Pare Mountains, NE Tanzania) and *quarta* (NE Zimbabwe and Mt Gorongoza, Mozambique). The locations of material sequenced and types of material used are summarised in Fig. 2.1 and Appendix I.

DNA extraction

DNA was extracted by digesting samples (0.01 – 0.02 g of ground tissue or 15 – 20 μl of blood) in 500 μl amniocyte buffer (50mM Tris, pH 7.6, 100mM NaCl, 1m EDTA, pH8.0, 0.5% SDS) with 0.5mg proteinase K (Roche Diagnostics) at 55°C for 12 – 24 hours. This was followed by RNA digestion with 0.1mg RNase A (Roche Diagnostics) at 37°C for 1 hour. Samples were then

extracted three times with phenol and once with a 24:1 solution of chloroform:isoamyl alcohol (Sambrook *et al.* 1989) solution and total DNA precipitated overnight at –20°C with 0.1 volumes 3M sodium acetate and 2 volumes 96% ethanol. The DNA pellets were collected in a tabletop microcentrifuge at 13000rpm for 30min. This was followed by a 70% EtOH wash whereafter the pellet was collected by spinning at 13000rpm for 30min and resuspended in 50µl Sabax® (Adcock Ingram) water preheated to 37°C and then stored at -20°C. Attempts at extracting sufficient concentrations of high quality DNA from samples stored in the 100% EtOH/formalin mixture via the Qiagen DNeasy tissue kit (Qiagen Inc), a protocol by (FM Catzeflis pers. comm.) and the method of Shedlock *et al.* (1997) were largely unsuccessful. Attempts to extract DNA from foot-scrapings followed the methods of Mundy *et al.* (1997).

PCR amplifications & primer design

Roughly 50-100 ng of DNA was used in the Polymerase Chain Reaction (PCR; Saiki *et al.* 1988) amplifications. Mitochondrial DNA fragments containing parts of the cytochrome *b* gene were amplified using several oligonucleotide primers (Table 2.1). Double-stranded amplifications were performed in a total reaction volume of 50µl using 5µl 10×reaction buffer, 2mM of each of the four nucleotides (dNTP's; Promega), 2.0 mM $MgCl_2$, 1.5U Super-therm® DNA polymerase (Southern Cross Biotechnology) and 50 pmol of each of the two primers. Reaction mixtures were subjected to 35 cycles of denaturing at 94°C (30 s), annealing at 53 to 61°C (45 s), and extension at 72°C (45 s) in a Geneamp® PCR System 9700 (Applied Biosystems). The first cycle was preceded by an initial denaturation of 5 min at 94°C and the last cycle was followed by a final extension of 7 min at 72°C. Negative controls were included in all PCR's.

Success of PCR reactions were checked on 1.0% agarose (Promega) gels, stained with ethidium bromide, before purification. Products showing specific amplification were purified directly using the High Pure™ PCR Product Purification Kit (Boehringer Mannheim). In the case of products showing non-specific amplification, the different sized bands were separated on a second 1.0% agarose gel. A 100bp molecular weight marker (Promega) was included to identify the correct band, which was excised from the gel, using a sterile scalpel blade, and purified according to the gel-extraction protocol provided by the manufacturers of the kit. All purifications were eluted in Sabax® water. DNA concentration was estimated spectrophotometrically or by running purifications against DNA of known concentration on ethidium bromide stained agarose gels.

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Table 2.1 Identities and sequences of primers utilized to amplify parts of the mitochondrial cytochrome *b* gene for *Apalis thoracica*. Primer names are numbered according to the position of the 3' base in the chicken mitochondrial genome (Desjardins & Morais 1990).

Primer name	General name	Location	Sequence of primer (5' to 3')	Forward/ reverse	Source
WBL11	L15237	Cytochrome *b*	GTCACAGGACTCCTACTAGC	forward	Constructed based on Warbler sequences from GenBank
H15696	H15696	Cytochrome *b*	AATAGGAAGTATCATTCGGGTTTGATG	reverse	Primer H15547 of Edwards *et al.* (1991)
H15915	H16064	Cytochrome *b*	CATTCTTTGGTTTACAAGAC	reverse	From Pääbo *et al.* (1988)
ND5L		ND5	TACCTAGGRTCHTTCGCCCT	forward	Constructed based on avian mtDNA sequences from GenBank
L15245		Cytochrome *b*	AAAGAAACCTGAAACACAGGAGT	forward	Modification of primer CB4a-L of Palumbi *et al.* (1991)
H15499		Cytochrome *b*	GGTTGTTTGAGCCTGATTC	reverse	Primer CBINT of Avise *et al.* (1994)

Sequencing

Both heavy and light strands were sequenced using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq ® DNA polymerase, FS (PE Applied Biosystems). Approximately 80-100ng purified template, 3.2pmol of the relevant primer and 4µl of the BigDye™ ready reaction mix was made up to 10µl with Sabax® and cycled in a Geneamp® PCR System 9700 (Applied Biosystems). Alternatively a mix of template, 1.8 picamol of the relevant primer and 2µl of the BigDye™ ready reaction mix was made up to 10µl with Sabax®. Cycling conditions followed the manufacturer's instructions and primers WBLL1 and H15696 were used to generate sequences.

Cycle sequencing products were purified using a modified NaAc precipitation method (PE Applied Biosystems). According to this method 10µl Sabax® sterile water, 2µl NaAc (3M) and 50µl 100% sequencing grade EtOH was added to a 10µl cycle sequencing reaction in a 500µl centrifuge tube, mixed and left on ice for 10min. DNA pellets were then collected by centrifugation at 13000rpm for 20-30min. The EtOH-supernatant containing unincorporated dyes was removed and the "pellet" washed once with 70% sequencing grade EtOH. A second centrifugation at 13000rpm for 15min followed. The 70% EtOH were removed and the pellets dried on a heating block at 60°C for 2min.

Following gel electrophoresis on ABI 377 or ABI 3100 automated sequencers (PE Applied Biosystems), electropherograms of light and heavy strand sequences were imported into Sequence Navigator™ version 1.0.1 and proof read. Consensus sequences were then aligned in Clustal X Multiple Sequence Alignment Program Version 1.63b (Thompson *et al.* 1997) and a Nexus file was created. Nucleotide sequences were translated to amino acid sequences in MacClade (Version 3.07, Maddison & Maddison 1992) in order to check for reading frame errors and termination codons.

Phylogenetic analysis

GenBank sequence data for the following taxa were included as outgroups for phylogenetic analysis: *Prinia bairdi* (AF 094617; Cibois *et al.* 1999) and *Cisticola juncidis* (AJ004314; Helbig *et al.* 1996). In addition one representative of *Apalis flavida*, Yellowbreasted Apalis, was sequenced and included as an outgroup. The number of variable positions was used to identify haplotypes and one representative of each haplotype was included in the analysis. Modeltest

Version 3.06 (Posada & Crandall 1998) as well as the maximum-likelihood (ML) method in PAUP* Version 4.0b10 (Swofford 1999) was used to estimate the best-fit model of DNA substitution, base frequencies, proportion of invariable sites (I) and the gamma shape distribution parameter. In addition, the transition: transversion ratio (ti:tv) was estimated in ML as well as the variability at each codon position by excluding constant characters in PAUP* Version 4.0b10 (Swofford 1999).

The distance method using the HKY85 model of nucleotide substitution (Hasegawa et al. 1985) was used to calculate the pairwise genetic distances among haplotypes. This model was selected as the model which best fit the data by maximum-likelihood (ML) and takes into account unequal base frequencies as well as the different rates for transversions versus transitions. The Mantel nonparametric test calculator Version 2.0 (Mantel 1967) was used to test the significance of the correlation between genetic distance and geographic distance. In order to detect saturation (Moore & DeFilippis 1997), scatter plots of percentage sequence divergence versus the number of pairwise transitions and transversions for each of the three codon positions were constructed. Saturation was thought to have occurred when DNA substitution levels off as percentage sequence divergence increases (Roy 1997).

Phylogenetic analysis was then performed in PAUP* Version 4.0b10. Evolutionary trees were constructed using maximum parsimony (MP; Hennig 1966) and neighbour-joining methods (NJ; Saitou & Nei 1987). Due to the size of the dataset, ML in PAUP* could not be used to generate bootstrap ML trees. The MP analysis was performed with unordered characters in addition to weighted analyses: (a) by resetting the character weights to the inverse of the variability at each codon position; (b) by weighting the characters according to the ti:tv ratio; (c) by weighting as described in (a) and (b) simultaneously; and (d) trees were reweighted according to the consistancy index (Farris 1969) until the number of trees remained the same for three consecutive reweightings thereafter the strict consensus tree was calculated. Secondly, distance analysis was performed by implementing the best-fit model of DNA substitution. Finally the robustness of all evolutionary trees was assessed through the bootstrap procedure (Felsenstein 1985), using 1000 random re-samplings. Bremer support/decay indices (Bremer 1988) were calculated for all nodes using AutoDecay v. 4.0 (Eriksson 1998) on the heuristic tree that was reweighted to the consistency index.

Molecular clock

The molecular clock has been criticised and it is clear that there are some problems with the concept and that it should be applied with caution (Avise 1994). Problems include variation in substitution rates among genes and between clades (Li 1993a, 1993b; Hillis & Moritz 1990) and the poor fossil record for birds that prevent calibration of the molecular clock for the particular clade being studied (Van Tuinen & Hedges 2001). Despite these complications the molecular clock concept allows estimation of approximate divergence times (Brown *et al.* 1979; Shields & Wilson 1987).

A likelihood ratio test (Felsenstein 1988; also see Yang *et al.* 1995; Sanderson 1998) was used to test the null hypothesis that the sequences were evolving at constant rates and therefore fit a molecular clock. The test was performed as follows: The likelihood score was estimated in PAUP* on a tree generated with the likelihood analysis using the no branch swapping option, with and without enforcement of a molecular clock (ti/tv ratio, base frequencies, proportion invariable sited and gamma shape parameter as estimated with likelihood). This resulted in two likelihood scores; the test statistic is equal to twice the ratio of the log likelihood scores, which is chi-square distributed with n-2 d.f., where n is the number of terminal taxa (Yang *et al.* 1995 also see application of test by Hibbett 2001; Piaggio *et al.* 2002). If there is no rate variation among lineages, a molecular clock calibrated at 2%/ Myr. (Shield & Wilson 1987; Tarr & Fleischer 1993; Fleischer *et al.* 1998) which is a rate generally applied for birds (Cicero & Johnson 1995; Helbig *et al.* 1995; Roy 1997; Voelker 2002) could be employed to calculate rough estimates of times of divergence.

Results

Sequence characteristics

There was no contamination in the negative controls of the PCR reactions except for the museum material. The results of the foot scraping samples were not included in the final analyses because not all samples were amplified, and some that were, resulted in short sequences (*ca* 200bp) which were contaminated through sharing of extraction solutions and pipettes. This is a reminder of the need to be extremely cautious when working with ancient DNA samples.

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Six hundred base pairs of the cytochrome *b* gene (Appendix II) were analysed for 26 ingroup and an additional 3 outgroup individuals. Of the 600bp analyzed, 159 characters were variable of which 24 were located at codon position one, four at position two and 131 at the third position. The variable positions defined 24 haplotypes for *A. thoracica* (Table 2.2) of which only one representative of each was included in all subsequent analyses. The data contained 104 parsimony informative characters and a proportion of 0.658 invariable sites with a gamma shape parameter of 1.634. Transitions outnumbered transversions 6:1 and the base frequencies of A and C (0.3120 and 0.3469 respectively) were much higher than for G and T (0.1374 and 0.2037 respectively).

Appendix III shows the HKY85 pairwise distances among haplotypes ranging from 0.2-8.1% within *A. thoracica* and from 8.4 to 16.7% between *A. thoracica* and the outgroup taxa. When the distances were plotted against the number of transitions and transversions, there was no evidence of saturation in any of the codon positions (Fig. 2.2).

Phylogenetic analysis

Tree topology was generally in agreement between the different analyses with the exception of the two most basal subspecies, *griceiceps* and *uluguru*, which swapped positions (Fig. 2.3). Most of the relationships among the taxa were well resolved with high bootstrap values. The decay index values agreed better with some bootstrap values than with others (Fig. 2.3). The cytochrome *b* data provided good support for the monophyly of the *A. thoracica* species complex. Many of the internal branch lengths were short and all outgroups grouped basal.

Phylogenetic analysis recognised 11 distinct mtDNA clades that were well supported by bootstrap values (Fig. 2.3). Keeping in mind the potential limitations of the current sample availability, especially the absence of sequencing data for subspecies *pareensis* and *quarta*, the results provided good support (Fig. 2.3) for morphologically defined subspecies *griseopyga*, *lynesi*, *fuscigularis*, *uluguru* and *griseiceps* as distinct lineages.

The majority of the clades included more than one neighbouring subspecies and were well supported (Fig. 2.3). The three Cape subspecies *A. t. capensis* Roberts 1936 (Ann. Transv. Mus. 18: 306), *A.t. claudei* (*A. claudei)* Sclater 1910 (Bull. Br. Orn. Club 27: 15) and *A. t. thoracica* (*Motacilla thoracica*) Shaw & Nodder 1811 (Nat. Misc. 22 pl. 969) grouped together with 98% bootstrap support (the last name has priority). The two KwaZulu-Natal subspecies *A. t. venusta* (*A. venusta*) Gunning & Roberts 1911 (Ann. Transv. Mus. 3: 116) and *A. t. darglensis* (*A. darglensis*) Gunning & Roberts 1911 (Ann. Transv. Mus. 3: 117) grouped together with 100%

bootstrap. In addidtion the *darglesis* individual made *venusta* paraphyletic. These molecular results support the inclusion of *darglesis* in *venusta* by Urban *et al.* (1997) and the clade will be referred to as clade *venusta*.

Subspecies *A. t. spelonkensis* (*A. spelonkensis*) Gunning & Roberts 1911 (Ann. Transv. Mus. 3: 116) and *A. t. flaviventris* (*A. flaviventris*) Gunning & Roberts 1911 (Ann. Transv. Mus. 3: 117) grouped together with 99% bootstrap (the first name has priority) and were paraphyletic which does not support the distinction between them. There was less support for clade *drakensbergensis* (61%) containing subspecies *A. t. drakensbergensis* Roberts 1937 (Bull. Br. Orn. Club 57: 99) and *A. t. lebomboensis* Roberts 1931 (Ann. Transv. Mus. 14: 242). The sequence divergences within these clades were extremely low (0.2-0.8%) and one of the *venusta* individuals had an identical haplotype to a *darglensis* individual (Appendix III).

In the remaining two clades the sequence divergences among the members of the clades slightly exceeded 1%. Subspecies *A. t. rhodesiae* (*A. murina rhodesiae*) Gunning & Roberts 1911 (Ann. Transv. Mus. 3: 115), *A. t. arnoldi* (*A. griseiceps arnoldi*) Roberts 1936 (Ostich 7: 75) and *A. t. whitei* (*A. murina whitei*) Grant & Mackworth-Praed 1937 (Bull. Br. Orn. Club 57: 114) formed a monophyletic clade. The long branch length of race *arnoldi* suggested that it was distinct from the other two but since it grouped between them and was sister to *whitei* in the NJ analysis but sister to *rhodesiae* in the MP analysis, there can't be a distinction between these based on the current dataset and they will be treated as clade *rhodesiae*. The subspecies occurring near Lake Malawi, *A. t. flavigularis* (*A. flavigularis*) Shelly 1893 (Ibis 1893:16), *A. t. youngi* Kinnear 1936 (Bull. Br. Orn. Club 57: 8) and *A. t. murina* (*A. murina*) Reichenow 1904 (Ornith. Monatsber. 12: 28) formed a well-supported monophyletic clade (88% bootstrap support). Although race *flavigularis* had several unique mutations and appeared to be phylogenetically distinct it had a sister relationship with race *youngi* therefore all tree will be treated as clade *flavigularis*.

Table 2.3 shows the distances but regrouped into percentage sequence divergence between mtDNA clades. The sequence divergences among the clades were generally above 2% (lower limit 1.3-5.6%) and ranged up to 9.4% (upper limit 4.2-9.4%). The largest sequence divergences were between clades *griseopyga*, *thoracica* and *venusta* (southern South Africa) versus the remainder of the clades (4.6-8.1%). These three clades were more closely related to each other (3.0-4.7%) than to the remaining clades. Similarly the remaining clades were more closely related to each other (1.3-5.3%; Table 2.4).

Table 2.2 Aligned nucleotide sequences showing variable positions in the mtDNA cytochrome *b* gene for *Apalis thoracica* and outgroups: *Prinia bairdi*, *Cisticola juncidis* and *Apalis flavida*. Dots indicate identity with the first sequence. Undetermined nucleotides are marked by '?'.

```
                                       11111111111111111111111112222222222222222222222222233333333333333333333333333333333334444444444444444444444444444445555555555555555555555555555556
                111112233334455566789990112333445666677788899900011223345677788899000112233344455566777888899990001111222333445677888899990001111222233344445556667889990
Taxon/Node   13690256847036958047065403924730584732689457069258147095847927037925947039284703925814736235801470369028147803656814654706892689147147924581250179289147928157 0
-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------
griseop 1    TCCAACCAGATCACCATGTATCATCCACCCCTACACCCCATAACCATCCAATATAGAAATACCCACAGCAAGACATTCAACACCCTTACCACTCAGGACACCCCTTATCAAGCAACTCCCCCAGACCTACCCTCCCCTTGCCTGCTGCAACCATCCAC
griseop 2    .................A............................................................................................................................................
capens  1    .........C...........C....T........T.CG...C....C.A.A...............A........T.....C........A..............................T...T...................T..
capens  2    .........C...........C....T........T.CG...C....C.A.A...............A........T.....C........A...............A..............T...T...................T..
cap/clan     ???......C..T........C....T........T.C.G..C....C.A.A...............A........T.....C........A..............................T...T.................?????
clandei      .........C...........C....T........T.C....C....C.A.A...............A........T.....C........A..............................T...T.................C.T..
thoraci 1    .........C...........C....T........T.C....C....C.A.A...............A........T.....C...T..A...............A..............T...T..................????
venusta 1    .........C...........C...TT........T..G..A...GGC.AG..........TG........CC...T....CC..............T....C.....AT................................G.T......T
venu/dargl#
..........C...........C...TT........T..G..A...GGC.AG..........T..........CC...T....CC..............T....C.....AT................................G.T......T
drakens 1    .....T...C...........C....T.........CC..T....GGC.A.............T.......C.........C.C....C.GAA.........C...G.A.......T.T.T.....G.....T.....................CT...
drak2/leb*   .....T...C...........C....T.........CC..T....G.C.A.............T.......C.........C.C....C.GAA.........C...G.A.......T.T.T.....G.....T.....................CT...
spelonk 1    ?TG.......CA...........C....T.........CC..T..C..GGC.A.A...C.........T.......C.........C.C....C.GAA.T......C...G..........T.T.T.....G...................C......GCT...
spelonk 2    .........CA...........C....T.........CC..T..C..GGC.A.A...C.........T.......C.........C.C....C..AA.T......C...G..........T.T.T.....G...................C.T....GCT...
flaviven     ..G..T....CA...........C....T.........CC..T..C..GGC.A.....C.........T.......C.........C.C....C.GAA.T......C...G..........T.T.T.....G...........?????????????????
rhodesia     .........C.G...........C....T.........CC........T.C.A..................T......C.....T.TC.C....C..AA.......CC...............TT.T.T.....G................A......CT...
arnoldi      ...C......C.G.....C....C.T...T.........CC........T.C.G.........A.....TG.....C.....T.TC.C....C..AA..G.....C...............TT.T.T....?G..A..........T.....T.....CT...
whitei       .........C.G...........C....T.........CC........G.C.A.........A.....TG.....C.......TC.C..G.C.GAA..G....CC...............TT.T.T.....G...........................CT...
lynesi       .........C.......C....C....T.........CC........G.C.A...G.C.........C......C.........C.C....C.GAA..G....CC.........G....T.T.T.....G...........................CT...
flavigul     .........C.......C....C....T.........CC..........C.G.A...CG..A.....TG...GCC....G...C.C..G.C.GAA.TC.....C.....A.......T.?.T.G...G..A......C.TT...?.T.....C....
youngi       .........C.......C....C....T.........CC........G.C.G.A...CG........TG....CC.........C.C....C.GAA..C....CC.....A.......T.T.T.G...G..........C.T.............C....
uluguru      ?????????.C.......C...CC.....T......TT.CC..T.....G.C.G.A...CG.....GA..G..T.CC.........C.C..G.C.GAA..T....CC..T..A....C..T...T.....G...............T..........C....
murina       .........C.......C....C....T.........CC........G.C.G.A...CG........TG....CC.........C.C..G.C.GAA..C....CC.....A.......T.T.T.G...G..........C.T.............C....
griseic      .........C.....C.C....C....T.........CC..T.....G.C.A.A..GCG.....GA.TG....CC......T.C.C..GTC..AA..C....CC.....A....C..T.T.T.....G...........................C....
fuscigul     .........C.......C....C....T.C.......CCG.....T.G.C.A.A...CG.........T.....CC.........C.C..G.C..AA..C....CC.....A....C..T.TTTA....G...........................C....
A.flavida    G...GT..ATC..T..C.C..T.C......T...G.........G..T..CC.G.AG.GC...A.T.A.......CCTC....T.CCC..C.C..A.G.C....ACG....A....C.....T.GA...CG..A....TACA..C...A....T.C...T
Cisticola    ...CGT..A.C...A.C.C.CTC....TA......G.?.CCT..T.CT...CCG.A..GC..T..T.AT..A.T.A.T.C...TTCCCTTG....AA..C.TA.CC.AA..AA......T....A.T.A...AC..TTA.A.ACTT.C..T.T.C....
Prinia       ...C..TGA.C.GT.GC.CG..GCT.TTA.T.CT...A.CC...GGA.TG.CCG....GC.A.AG..ATC.AC..AC...T...T.C.G.G.CT.AA.AC...ACC.AT.CA.T.TCT.TT...A..GA..TACATT..CAT.CTA....C...C..GT
```

**drakensbergensis* 2 (*drakens 2*) and *lebomboensis* (*lebombo*) were identical in sequence (Appendix III) therefore only one representative was included and called *drak2/leb*
#*venusta 2* (venusta 2) and *darglensis (darglen)* were identical in sequence (Appendix III) therefore only one representative was included and called *venu/dargl*

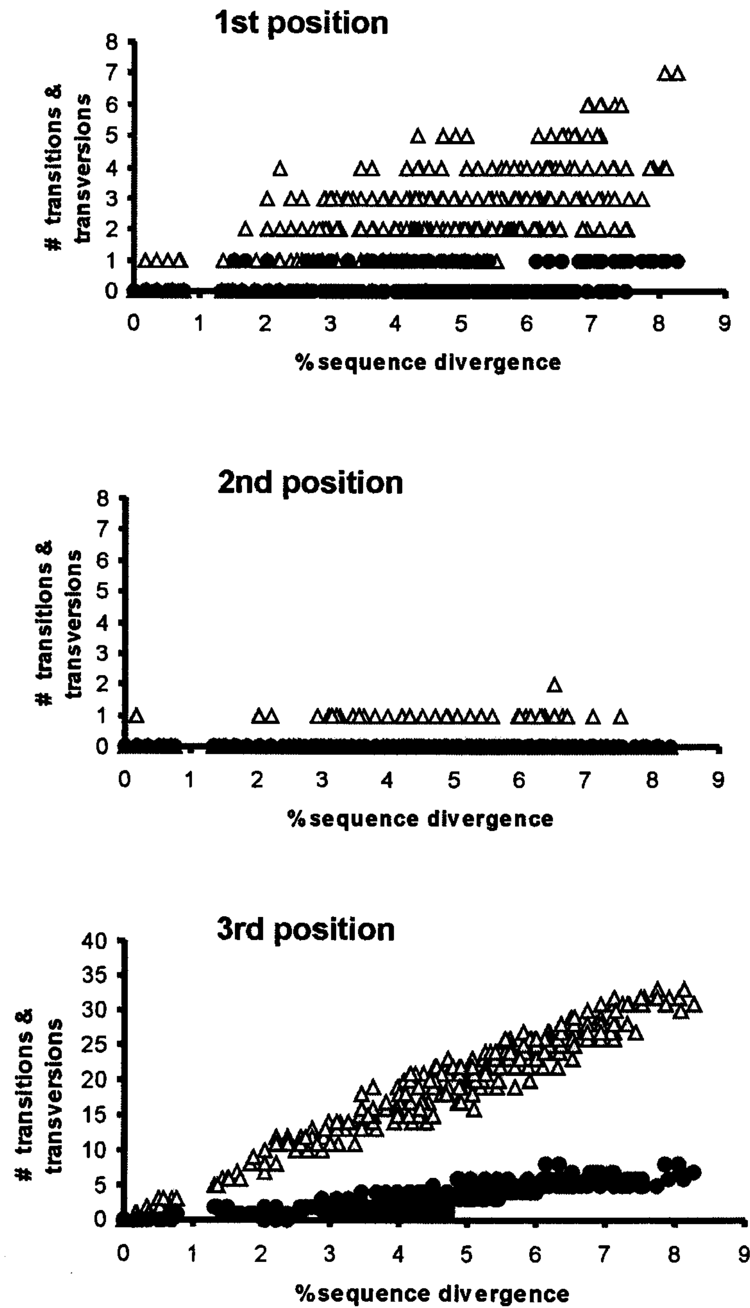

Figure 2.2 Saturation curves of first, second and third position transitions and transversions vs. percentage sequence divergence per pairwise comparison of haplotypes of the *Apalis thoracica* complex for cytochrome *b*. Open triangles represent transitions and closed circles represent transversions.

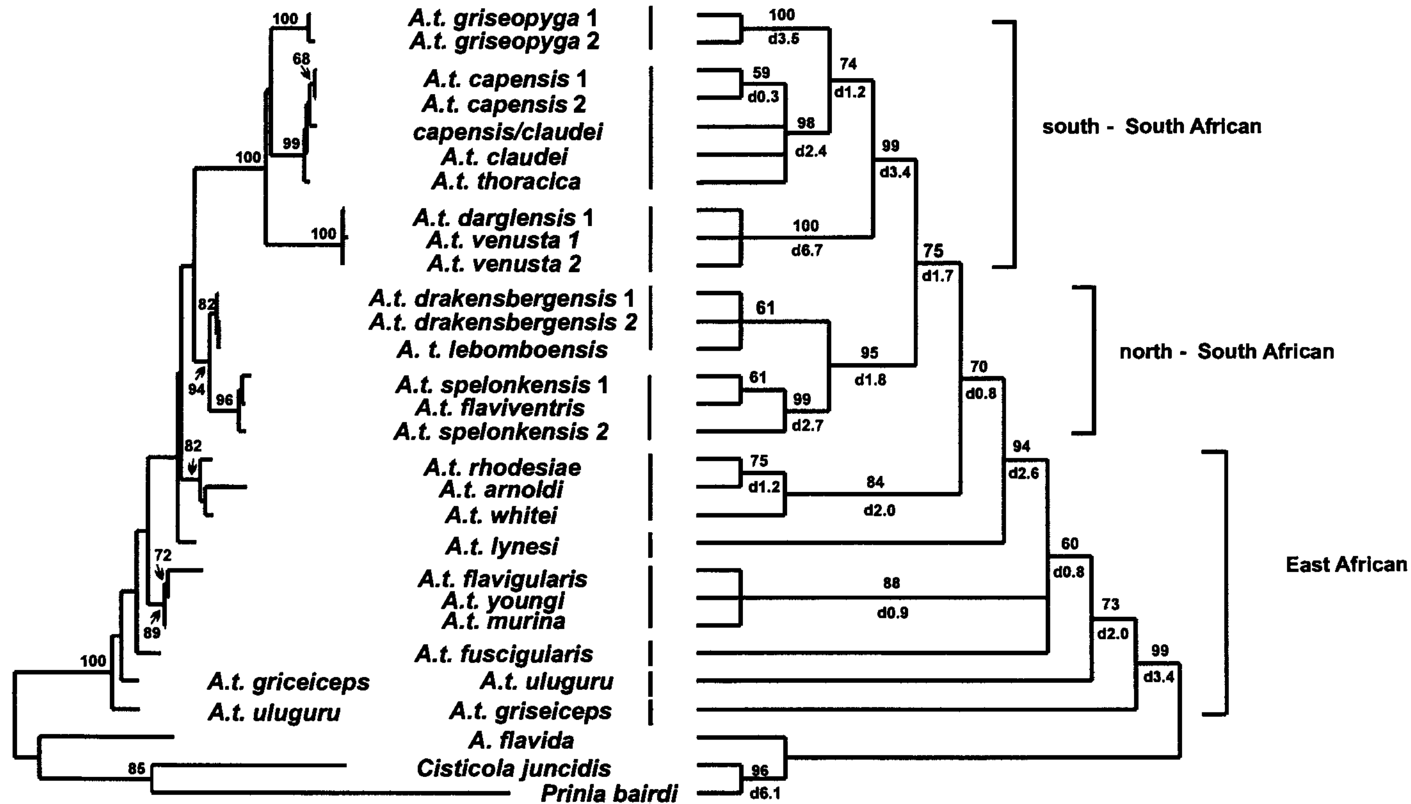

Figure 2.3 Reconstruction of the molecular phylogeny of the *Apalis thoracica* complex based on cytochrome *b* sequences and using three outgroups including another *Apalis* warbler (*A. flavida*) and two potentially related sylviids (*Cisticola juncidis* and *Prinia bairdi*). All subspecies except *pareensis* and *quarta* were sequenced. The neighbour-joining phylogram is shown on the left with bootstrap values (1000 replicates). Parsimony cladogram with bootstrap values (1000 replicates) and Bremer support/ decay indexes for the branching nodes is shown to the right. Bremer support values were calculated on a tree that was reweighted to the consistency index.

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Table 2.3 Percentage sequence divergence between molecularly defined lineages based on 600bp of the mitochondrial cytochrome *b* gene. The lined box includes sequence divergences between the south-South African group (lineages *griseopyga*, *thoracica* and *venusta*) versus the remaining lineages.

Lineage	*griseopyga*	*thoracica*	*venusta*	*drakensbergensis*	*spelonkensis*	*whitei*	*lynesi*	*murina*	*fuscigularis*	*uluguru*	*griseiceps*	*arnoldi*	*flavigularis*
griseopyga	–	2.97 - 3.27	4.35 - 4.71	4.88 - 5.42	5.80 - 6.38	4.87 - 5.60	5.06 - 5.24	5.98 - 6.35	6.35 - 6.54	7.32 - 7.52	6.92 - 7.11	6.15 - 6.34	6.92 - 7.12
thoracica		–	3.99 - 4.72	4.61 - 5.26	5.25 - 6.20	5.06 - 6.17	5.25 - 5.81	5.99 - 6.64	5.99 - 6.65	7.34 - 7.74	6.50 - 7.13	6.53 - 7.10	6.94 - 7.32
venusta			–	5.62 - 5.99	6.31 - 7.33	6.35 - 6.92	6.36 - 6.55	6.73 - 7.11	6.54 - 6.73	7.91 - 8.11	7.30 - 7.50	7.86 - 8.05	8.08 - 8.27
drakensbergensis				–	1.32 - 2.22	2.56 - 2.89	2.21 - 2.50	3.13 - 3.62	3.80 - 4.20	4.29 - 4.62	3.98 - 4.42	3.93 - 4.15	4.40 - 4.90
spelonkensis					–	3.44 - 3.81	2.89 - 3.09	4.16 - 4.49	4.34 - 4.72	5.04 - 5.41	4.52 - 4.49	4.51 - 5.25	4.70 - 5.09
whitei						–	1.87 - 2.21	2.74 - 3.62	3.45 - 3.63	4.46 - 5.40	3.62 - 4.17	2.04	4.0 - 5.09
lynesi							–	2.39 - 2.56	2.92	4.09	3.45	3.26	4.17
murina								–	2.04 - 2.22	2.82 - 3.00	2.22 - 2.39	3.97 - 4.15	1.52 - 1.69
fuscigularis									–	3.55	2.22	4.88	3.63
uluguru										–	2.64	5.77	4.3
griseiceps											–	5.06	3.81
arnoldi												–	3.82
flavigularis													

Clades *griseopyga* and *thoracica* showed a sister relationship (<50/74) and were more closely related to each other (3.0-3.2%) than either was to the basal grouping clade *venusta* (4.0-4.7%). Together all three clades formed a well-supported monophylum which will be referred to as the south-South African group (sSA). This sSA group in particular as well as the three clades within had extremely long branch lengths (Fig. 2.3). Similarly clades *drakensbergensis* and *spelonkensis* formed a well-supported monophylum (Fig. 2.3), the north-South African (nSA) group. The remaining clades grouped progressively more basal with a decrease in latitude. There seemed to be a general increase in sequence divergence (Appendix III) with increase in geographic distance with the largest sequence divergences between the most southern and most northern taxa. No correlation was however found between genetic distance and geographic distance when performing the Mantel test (Critical value at 0.050 was 1.645). This implies that the isolation by distance model (Wright 1943; Slatkin & Maddison 1990) cannot be invoked to explain the geographic distribution of genetic diversity in *A. thoracica*.

Table 2.4 gives a summary of the most significant differences in plumage among the subspecies. In *A. thoracica* the geographic variation in morphology mainly affects the coloration of the upper and under-parts, especially the top of the head and the degree to which the ventral surfaces are suffused with lipochrome (Lawson 1965). The combination of a white throat and chin with a black throat band, yellow belly, brown forehead and crown and green on the upper-parts is present in most lineages and may be plesiomorphic traits. The completely black throat, chin to upper breast in race *lynesi* as well as the blackish chin and throat that merges with the black breast band of race *fuscigularis* seem to have evolved independently. The rusty brown colour in the upper-parts, throat and flanks of race *claudei* may be the result of an increase of melanin in the pigmentation of the plumage for populations in moist and humid habitats (Lawson 1965). In *flavigularis* the white chin and throat disappeared independently and was replaced by a complete, bright yellow chin, throat and belly. The green upper-parts and yellow under-parts were independently lost in *griseopyga* and *capensis*. Both are a pale grey color above including on the forehead and crown with white below and greyish flanks. In fact, most of the South African races have a grey forehead and crown except *drakensbergensis*. This race exhibits green that evolved independently on the forehead and crown. The pale colour is thought to be the result of a phenomenon seen in other avifauna in South Africa where the populations showing least lipochrome occur along the West Coast of the Cape Province (Lawson 1965). A clinal trend appears in the colour of the under-parts including the throat which become progressively more yellow as one moves east and north (reflects an increase in lipochrome; Lawson 1965) and there is an intrusion of green on the upper-parts seen in most taxa further north (Table 2.4; Urban *et al.* 1997).

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Table 2.4 Morphological descriptions of morphologically defined subspecies of *Apalis thoracica*. Molecularly defined clades are also indicated

Clade	Subspecies	Morphological description Forehead & crown	Upperparts	Underparts	Wing & Tail
griseopyga	*griseopyga*	pale blue grey; lipochrome virtually absent from plumage	pale blue grey	white below; greyish flanks	
	capensis	grey	grey	white below; greyish flanks	
thoracica	*clandei*		rusty olive brown	throat pale rusty buff; belly white, flanks tinged olive brown	
	thoracica	light grey	olive green back	throat white, belly primrose yellow	
	venusta	grey	mantle & back olive green	belly more yellow than previous	
venusta	*darglensis*	grey	mantle & back olive green	belly more yellow than previous	
	drakensbergensis	green	back yellowish-green	incl. throat more yellow	
drakensbergensis					
	lebomboensis				
	flaviventris	grey	olive green	more yellow than previous, less than next	
spelonkensis	*spelonkensis*	greyish brown	back green	incl. lower troat bright cream yellow	
arnoldi	*arnoldi*	incl. cheeks, ear coverts sooty brown; dark loral streak	nape, mantle, back to uppettail coverts dark moss green	chin, throat off-white; breast, belly, flanks to undertail-coverts strongly	
whitei	*rhodesiae*	crown paler, greayer than previous	back greyish, faintly washed pale green	chin & throat cream white belly & flanks washed pale yellow	
whitei	*whitei*	crown brownish	green	belly washed yellow	

Table 2.4 (continued)

Clade	Subspecies	Morphological description Forehead & crown	Upperparts	Underparts	Wing & Tail
lynesi	*lynesi*	male		chin, throat to upper breast deep black (obscuring breast band); belly olive yellow brighter in center; flanks & undertail-coverts deep olive	
		female		chin, throat, upperbreast sooty; center of breast dark grey (vestigal breast band); sides washed green; flanks olive	
flavigularis	*flavigularis*	male - black with greenish wash ear-coverts dark grey female - crown sooty brown; black loral streak	mantle, back bright green; wing green	bright yellow under wing-coverts; yellowish flanks washed olive	tail black; outer webs of feathers margined green
murina	*youngi*	crown brown	back to rump, upper tail-coverts clear slate grey washed olive	belly to under tail-coverts, flanks white	
murina	*murina*	crown to nape brown	back grey; rump grey green	lower belly yellow; flanks strongly washed olive-green	
fuscigularis	*fuscigularis*	top & sides of head earth brown; ear-coverts lighter than *arnoldi*	back blackish-grey; rump, upper tail-coverts dark green wash	chin, throat blackish merging with dark breast band; belly, flanks, under tail-coverts off-white to sooty grey	wings blackish-grey; T5-T6 white with dusky margins; T4 white tipped
uluguru	*uluguru*	dark sooty brown	green	chin, throat white with grey feather bases; belly, flanks bright yellow	tail blackish-grey; white restricted to outer half of outer webs and tips of T5-T6
gricseiceps	*gricseiceps*	top, sides of head brown	back green	chin, throat, immediately below breast band white, belly, flanks bright yellow	

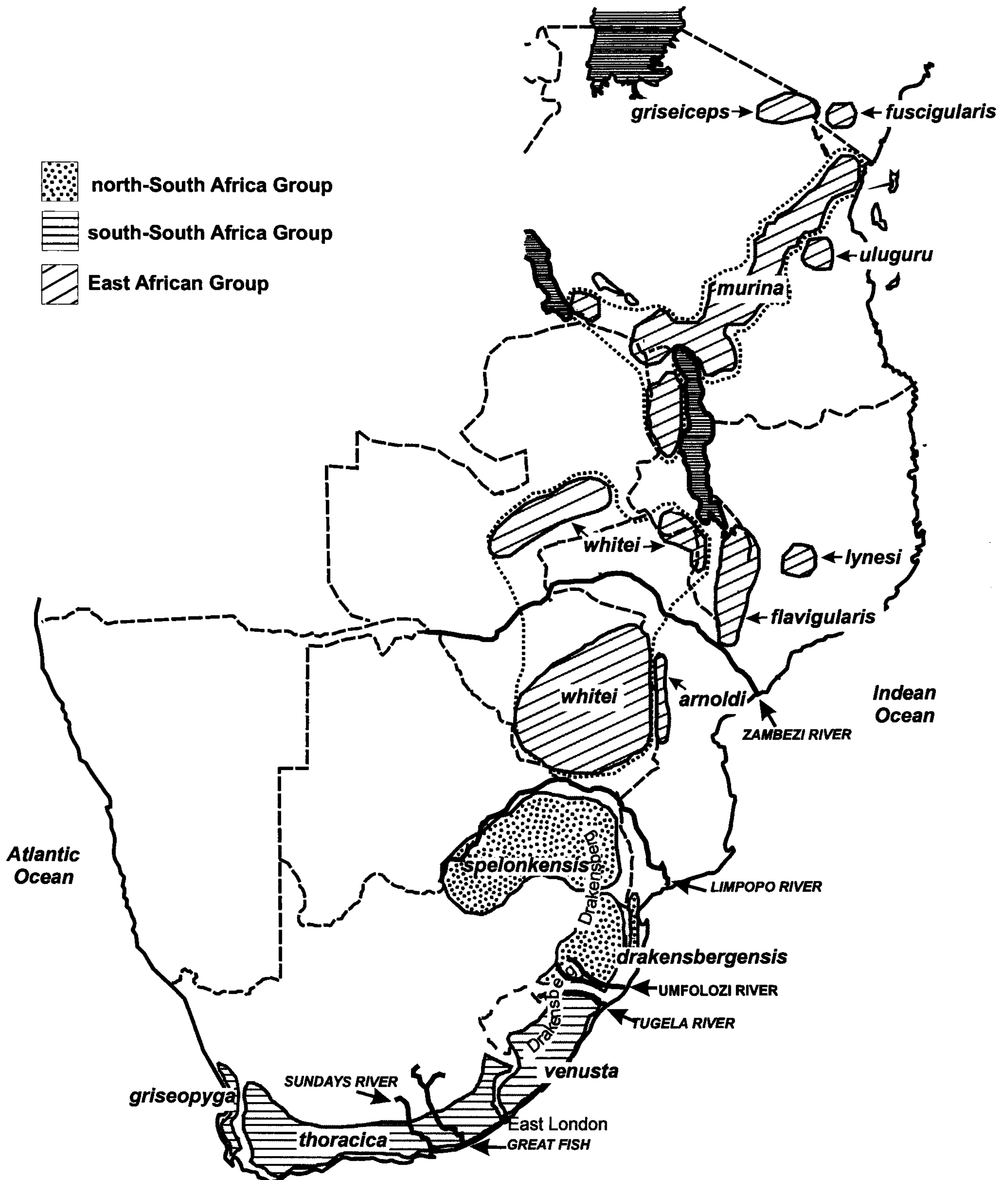

Figure 2.4 Geographic distributions of the 13 molecularly defined (cytochrome *b*) clades within the *Apalis thoracica* complex. Of the 21 subspecies all but races *pareensis* and *quarta* were included. Three geographic groups were identified: south-South African group, north-South African group and East African group.

The geographic distributions of the molecularly defined lineages as well as the groupings of the sSA, nSA and EA groups are shown in Fig. 2.4. The sSA group is more or less contiguously distributed from the Western Cape to the midlands and coastal forests of KwaZulu-Natal south of the Tugela River (Fig. 2.4). Although based on very few samples there seemed to be a molecular break within the sSA group between lineage *venusta* versus lineage *griseopyga* and *thoracica* which was located near East London. The geographic range of the nSA group seems to be contiguous with the sSA group and extends from the Tugela River northward to the mountains south of the Limpopo River Valley. The clades within the East African group occur in fragmented montane habitats north of the Limpopo River.

Molecular clock

When the log-likelihood ratio test of clock-like evolution was performed on the full data set, the molecular clock was rejected (Chi-Square = 73.2, d.f.= 27). A similar result was obtained when a data set with only *A. flavida* as the outgroup (excluding *Prinia* and *Cettia*) was subjected to the same test. Two alternative data sets were then subjected to the log-ratio test: (a) *Cettia*, *Prinia* as well as the lineages occurring north of the Tugela River excluded; (b) *Cettia*, *Prinia* as well as the lineages occurring south of the Tugela River excluded. For both these data sets the molecular clock was accepted (Chi-Square = 19.8, d.f.= 15 and Chi-Square = 8.3, d.f.= 9 respectively). Consequently the timing of divergence events was estimated within the lineages south of the Tugela and alternatively among lineages north of the Tugela. Rough estimates of times of divergence among the molecularly defined clades are shown in Appendix IV. Most of the divergence times between lineages dated to the Pliocene with the exception of clade *spelonkensis* versus *drakensbergensis*; *arnoldi* versus *whitei* and *flavigularis* versus *murina* (Fig. 2.5). These divergences were estimated to be more recent (Pleistocene). All estimated times of divergence within lineages dated to the Plio-Pleistocene or Pleistocene (Fig. 2.5). Although a direct comparison between the lineages south and those north of the Tugela River could not be made, the former group seems to be old (Pliocene) and the split between the two groups most probably dates to the Pliocene.

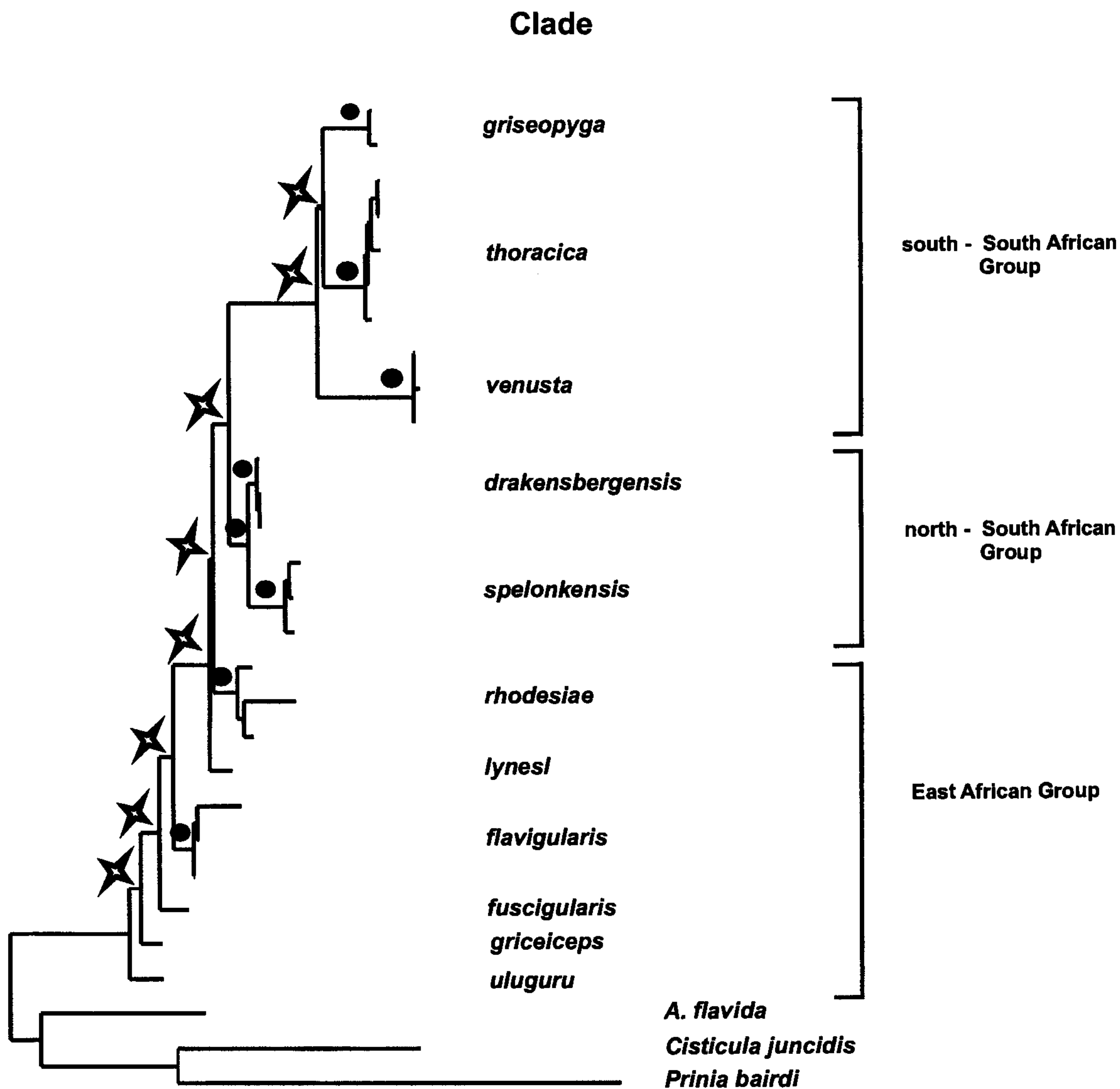

Figure 2.5 Neigbour-joining tree showing a reconstruction of the molecular phylogeny of the *Apalis thoracica* complex based on cytochrome *b* sequences. Another member of the genus (*A. flavida*) and two potentially related sylviids (*Cisticola juncidis* and *Prinia bairdi*) were included as outgroups. All subspecies except *pareensis* and *quarta* were sequenced. The 13 molecularly defined clades as well as the three major groups are indicated. Rough estimates of the time of divergence between taxa were made by employing a molecular clock calibrated at 2%/Myr. Stars represent Pliocene radiations and closed circles represent Plio-Pleistocene or Pleistocene radiations.

Discussion

Phylogeny

Based on the current dataset, which includes only one other Apalis species, *A. thoracica* formed a well-supported monophylum with short internal branch lengths indicating a fairly rapid radiation into 11 well supported distinct mtDNA clades. The validity of subspecies *griseopyga*, *griseiceps*, *lynesi*, *fuscigularis* and *uluguru* was supported on a molecular level by their genetic distinctiveness (Fig. 2.3), as well as by the distinctive plumage of the latter three (Urban *et al.* 1997; Table 2.4) with *lynesi* and *fuscigularis* treated as species by BirdLife International (2000).

Among the multimembered clades the sequence divergence values within lineages *thoracica*, *venusta*, *spelonkensis* and *drakensbergensis* were typical of values found within recognised subspecies (Johns & Avise 1998), providing no molecular support for the distinction among the subspecies within these clades. Members of lineages *venusta*, *spelonkensis* and *drakensbergensis* were very similar in plumage with subtle differences (Table 2.4). The members of lineage *thoracica* are however very different in plumage (Table 2.4). Within the lineage the most westerly race, *griseopyga*, exhibits an absence of lipochrome, typical of avian populations that occur along the West Coast of the Cape Province (Lawson 1965). The centrally distributed race *claudei* shows a brown coloration in plumage that reflects an increase of melanin in the pigmentation of the plumage, which is seen in many avian populations living in moist and humid habitats (Lawson 1965). *Apalis t. thoracica* is the most western race within the lineage and forms the first link in a clinal trend involving an intrusion of green on the back as well as yellow on the belly which extends further north.

Similar to the patterns in the Mountain Greenbul (*Andropadus tephrolaemus*; Roy *et al.* 1998) there is little concordance between morphological characters (plumage color) and genetically inferred phylogenetic relationships in *A. thoracica*. In general the molecular divergences and deep phylogeographic breaks within the contiguously distributed South African clades (nSA and sSA groups) did not correspond with morphological differences or breaks of the same magnitude (Fig. 2.3; Table 2.4).

The level of sequence divergence between *arnoldi* and the other members of the clade *rhodesiae* (Fig. 2.3) was within the range of species (Johns & Avise 1998). *Apalis t. arnoldi* is also morphologically distinct, with dark moss green upper-parts (darker than *whitei* and not as grey as *rhodesiae*) and brighter yellow under-parts. Race *arnoldi* was however sister to *whitei* in the NJ

analysis but sister to *rhodesiae* in the MP analysis and fell between *rhodesia* and *whitei* on both trees (Fig. 2.3). The specific sample of *arnoldi* was collected in an area of intergradation with *rhodesiae* (NW of Mutare; PG Ryan pers. comm.) and it would therefore be important in future research to compare specimens from the core of *arnoldi's* range (Vumba) to this sequence from NW of Mutare. For these reasons, based on the current dataset, these should be treated as belonging to the same clade, clade *rhodesiae*.

Similarly race *flavigularis* was distinct from *murina* and *youngi* and had several unique mutations that appeared to be recently derived (Fig. 2.3). It is the only race with entirely bright yellow under-parts (apart from the black throat band, Urban *et al.* 1997) and has consequently been treated as a species 'pending resolution of its taxonomy' by BirdLife International (2000). Race *flavigularis* however had a sister relationship with *youngi* and therefore the relationship among these tree species needs further investigation.

Lineages *griseopyga*, *thoracica*, *venusta*, *drakensbergensis* and *spelonkensis* are distributed more or less contiguously from the Western Cape to the Soutpansberg range in the north of South Africa (Harrison *et al.* 1997; Fig. 2.4), with a large possibility of gene flow between taxa. North of the Limpopo River however the clades are more isolated with little if any gene flow among taxa. Consequently larger levels of sequence divergence were expected among the isolated lineages than among the contiguous clades. This was however not the case. The genetic distances among the sSA and EA groups were roughly equal. In addition a possible deep phylogenetic break was found within the contiguously distributed clades (sSA versus nSA) and three molecular groups could be identified, the sSA, nSA and EA groups. The possible deep genetic break between sSA and the nSA group contrasts the minor differences in plumage. The break in fact falls within a clinal trend in plumage and the only plumage change corresponding with the marked genetic divide in South African populations is in crown colour (green in *drakensbergensis* and grey in *venusta/darglensis*).

It is generally assumed that the phylogeographic breaks observed in the contiguously distributed lineages is a consequence of historical barriers to gene flow that have since disappeared or diminished. It is predicted that the strength of such a barrier will rapidly decrease with time after the barrier disappeared and phylogeographic breaks will not persist unless there is very low individual dispersal (Irwin 2002). There is however an alternative explanation. Phylogeographic breaks can develop within a contiguously distributed species in the absence of a barrier to gene

flow when the average individual dispersal distance and/or population size of a species is low (Smith & Wayne 1997; Irwin *et al.* 2001a; b; Irwin 2002). The likelihood of observing phylogeographic breaks increase with a decrease in dispersal distance and population sizes (Irwin 2002).

The south-South African group

The long branch length of the sSA group suggests that it has been isolated for a very long period, south of the Tugela River, with little or no gene flow with other lineages and that it has accumulated several unique mutations. The Tugela River curtails the ranges of several other birds e.g. *Tchagra australis, Batis fratrum, Nectarinia neergaardi* and *Apalis ruddi* (Oatley 1989; Harrison *et al.* 1997). The average sequence divergence between this group versus the remaining lineages is on the level found between species (Johns & Avise 1989). This distinctiveness warrants further investigation. From a conservation point of view, the recognition of the three distinct clades (*griseopyga*, *thoracica* and *venusta*) south of the Tugela River (supported by high decay index values) presents previously overlooked biodiversity within South Africa. The persistence of this biodiversity will only be secure if parts of their distributions are included in conservation areas. In addition, the recognition of a distinct west coast subspecies *A. t. griseopyga* is significant in the light of other unique west coast specific taxa (long billed lark complex, Ryan & Bloomer 1999). This suggests that the West Coast has had its own unique evolutionary history resulting in distinctive forms of different taxa in the area.

The possible deep genetic break between lineage *thoracica* and *venusta,* which is located between East London and Port Elizabeth (Fig. 2.4), is seen as a subspecific break in other forest species as well such as the Forest Canary and Lesser Doublecollered Sunbird (Clancey 1980; Harrison *et al.* 1997; Urban *et al.* 1997). Interestingly in the East London area there is a juxtaposition of three different avifaunas that occur in tropical lowland forest, Afromontane forest and tropical woodland. South of East London there are no tropical forests and montane birds are separated geographically (Vernon 1989). The area is characterised by an erratic local climate (Vernon 1989) which may have been more pronounced during glacial cycles. This may have caused instability in the forest birds, which seem to be in a state of flux between colonisation, persistence, local extinction, absence and recolonisation (Vernon 1989). This may prevent geneflow among members of the species.

Two other factors may have played a role in the deep genetic break between lineage *thoracica* and *venusta*. The Great Karoo (Nama Karoo biome) which lies between the Swartberg and the Great Escarpment creates a gap in the distributions of several montane species for example *Geocolaptes olivaceus*, Ground Woodpecker; *Aquila verreauxii*, Black Eagle; *Onychognathus nabouroup*, Palewinged Starling (Urban *et al.* 1997). In addition, the area between Port Elizabeth and East London is known to be one of the regions of floristic endemism in South Africa, the Albany center. It is a large transitional zone where five phytochoria (White 1983) meet and numerous rivers of which the larger ones, including the Great Fish River, cut broad arid valleys (Van Wyk & Smith 2001). Some of these rivers are known barriers to dispersal in the distribution of birds (Harrison *et al.* 1997). Only small pockets of Afromontane and coastal forest are present in this area (Low & Rebelo 1996). Lawes (1990) identified the same region between the forests of the eastern and southeastern Cape as constituting a major barrier preventing the migration of the Samango monkey (*Cercopithecus mitis*) down to the forests of the southern Cape (Bedford gap). Broad sampling in this area will assist verifying the existence and location of such a break in *A. thoracica* and it would be interesting to see if a similar pattern is present in other montane taxa.

The north-South African group

The nSA group seems to be isolated between the Tugela and Limpopo River Valleys. Both valleys presents a barrier to other birds as well (Winterbottom 1959; Benson *et al.* 1962; Harrison *et al.* 1997). In many species the forests of the Soutpansberg south through Pilgrims' Rest in Mpumalanga and on the Drakensberg escarpment form a subspecific unit that is separate from the KwaZulu-Natal midland forests to the south (e.g. Olive Bush Shrike, Forest Canary, Southern Double-collared Sunbird) and the Zimbabwe Eastern highland forests to the north (Barratt's Warbler, *Bradypterus barratti*; White-starred Robin, *Pogonocichla stellata*; Yellow-throated Woodland-warbler, *Phylloscopus ruficapillus* (Harrison *et al.* 1997). In A. *thoracica* the nSA group was more closely related to the EA clades than to the sSA group. It is not so surprising that the nSA group is closely related to the East African species since the highlands of eastern Zimbabwe (Chimanamani, Vumba and Inyanga mountains) are recognized as constituting an important link between the Afromontane forests to the north of the Zambezi Valley and south of the Limpopo Valley (Harrison *et al.* 1997). It does however place more emphasis on the interesting deep phylogenetic break between nSA and sSA as discussed above.

East African clades

There seems to be a phylogeographic break between the mountains of the Eastern Arc and the northern Tanzanian mountains (Mt Meru) not only in *A. thoracica* but also in African greenbuls (*Andropadus tephrolaemus*; Roy 1997), thrushes (*Turdus olivaceus sensu lato*; Bowie *et al.* in press) and double collared sunbirds (Bowie *et al.* in review). Unfortunately we could obtain no sequence data for *A. t. pareensis* from the Pare Mountains, which form an interrupted chain between the Usambara Mts. and Mt. Kilimanjaro. Among white-eyes, the Pares support an endemic taxon *Zosterops [poliogaster] winifredae,* linked to populations further north. There are examples of montane avifauna where populations on the Pare Mountains are closely linked with populations on the Usambara Mountains: *Turdus* thrushes (Bowie in press), double collared sunbirds (Bowie *et al.* in review), and *Andropadus* greenbuls (Roy *et al.* 1998). In double collared sunbirds these populations are further linked to populations on the Taita Hills whereas in *A. thoracica* and the genus *Turdus* (Bowie in press) the Taita Hills support endemic taxa. In *A. thoracica* populations from Mt. Meru and Mt. Kilimanjaro are ascribed to the same subspecies. In *Turdus* thrushes however populations on Mt. Meru, Mt. Kilimanjaro and the Kenyan Highlands belong to three different subspecies (Bowie in press) and in double collared sunbirds, populations on the more recently formed Kilimanjaro and adjacent peaks are derived from populations on the Kenyan highlands (Bowie *et al.* in review) congruent with Moreau's classification of montane forest avifauna (Moreau 1966). The molecular distinctiveness of the Uluguru Mountain population is not surprising because this isolated peak is home to a number of endemic birds as well as mammals and invertebrates (Collar & Stuart 1988).

One of the weaknesses of the present study was the paucity of samples from the Eastern Arc Mountains of Tanzania in general. It is conceivable that although most of the populations in the Eastern Arc are ascribed to *murina*, there may well be hidden diversity within this complex (see e.g. Roy 1997, Roy *et al.* 1998, Roy *et al.* 2001, Bowie *et al.* in review). The omission of representative samples for subspecies *quarta* is less important, because it is likely close to races *arnoldi* and *rhodesiae*.

Molecular clock and biogeographic hypothesis

From Fig. 2.3 and 2.4 it can be concluded that *A. thoracica* has had a complex history of vicariance and interchange between different montane areas. It is believed that most South African forest fauna, including birds, are derived from lowland and montane communities in East Africa (Winterbottom 1968, 1974; Oatley 1989Lawes 1990). It has also been speculated that the

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Uluguru, East Usambara and Udzungwa Mountains had humid forest continuously since the upper Tertiary (Fjeldså & Rabøl 1995) and provided stable environments for the persistence of taxa through climatic oscillations. Therefore the basal grouping of taxa *uluguru*, *griseiceps* and *fuscigularis* suggest East African origins for *A. thoracica* as well.

The progressive basal grouping of taxa, in correlation with a decrease in latitude suggest that *A. thoracica* colonised suitable habitat in a north to south direction, reminiscent of a stepping stone model or isolation by distance (Wright 1943; Slatkin & Maddison 1990) although not supported by the Manteltest results. Once it colonised an area it became isolated and diverged before moving further south and colonising another area. The fairly short internal branch lengths (Fig. 2.3) suggest that these colonisation events happened in fairly rapid succession during the Pliocene (Fig. 2.5) without any gene flow in the opposite direction. Further divergence within lineages occurred on the Plio-Pleistocene boundary as well as in the late Pleistocene (Fig. 2.5) which is suggestive of more than one southward colonisation event that excluded some populations.

Some lineages are more closely related to lineages that are geographically further (*lynesi* versus *whitei*; *whitei* versus *griceiceps*) than lineages that are geographically closer (*lynesi* versus *flavigularis*; *whitei* versus *flavigularis*). There is thus a lack of correlation between genetic distance with geographic distance between present montane forests. Roy *et al.* (1998) in reaction to a similar pattern in *Andropadus* greenbuls commented that this may indicate a correlation with either the sequence of break-up of connections between montane forests, or with successful dispersal events during dry and wet climatic periods of glacial and interglacials (Diamond & Hamilton 1980). Roy *et al.* (1998) suggested that more detail on historic dispersal events from mountain to mountain could be obtained by sequencing a more rapidly evolving gene for large numbers of samples.

There are two possible explanations for the deep phylogenetic break between the sSA group versus the rest: (a) the sSA group may have been isolated from the rest for a very long period although it is uncertain what the mechanism of such isolation may have been or (b) the genetic break may have resulted from a difference in rate of evolution between the sSA group versus the rest. The latter is supported by the results from the molecular clock estimations. From the molecular clock estimations it is clear that the EA and nSA lineages relative to each other and *A. flavida* are clocklike and that the sSA lineages relative to each other and *A flavida* are clocklike but that the two groups, EA and nSA verus sSA are not comparable.

Consistent with the findings of Klicka & Zink (1997, 1999) and Avise & Walker (1998) species radiations in *A. thoracica* predate the Pleistocene whereas divergences among geographically segregated conspecific populations and subspecies date to the Pleistocene (Roy 1997). The estimated times of divergence in *A. thoracica* are consistent with the hypothesis of Avise & Walker (1998) that many speciation events are of protracted nature (Klicka & Zink 1997) extending from a Pliocene origin to Pleistocene completion and that the latter epoch may have played an active role in initiating major phylogeographic separations within species (Avise *et al.* 1998). Interestingly, the dates of species separations do not coincide with the occurrence of the last rifting in East Africa 7 Million years ago (Ma) (Griffiths 1993) but rather post-date these events by3-5 Myr. This implies that these riftings did not play as significant a role in diversification of the *A. thoracica* complex as it did in the case of Akalats in the genus *Sheppardia* (Roy *et al.* 2001).

Possible cryptic species and taxonomic consequences

The mtDNA sequence data, although limited by sample size and incomplete geographic sampling, allow some tentative taxonomic conclusions. Of the 19 subspecies sampled, only 13 were genetically distinctive. Many of the sequence divergence values among the lineages exceeded 2.6% which is the highest value so far known between established subspecies within warblers (Helbig *et al.* 1995, *Phylloscopus* warblers). Clades *arnoldi* and *flavigularis* both have unique mutations and are morphologically distinct from the other members of their respective clades therefore under the phylogenetic species concept they can be recognised as species. In addition some of the sequence divergences especially between the sSA group and the remaining lineages (4.6-8.1%) are unlikely to occur within interbreeding populations and are in the range of values found between species within passerine genera (Helbig *et al.* 1995; Leisler *et al.* 1997; Helbig & Seibold 1999) and congeneric avian species in general (Johns & Avise 1998). These values are also well within the range that has been used to suggest the species status of taxa previously described as subspecies (Leisler *et al.* 1997; Helbig & Seibold 1999).

It is debatable whether all 11 taxa deserve species status. Considering the Phylogenetic Species Concept one can certainly make a case for their species status but, although phylogenetic information is sometimes used to define species status of taxa, species should not be delimited based on mtDNA data alone because it is maternally inherited and gene trees do not necessarily reflect species trees as past hybridisation may distort results (Zink & McKintrick 1995).

Combining evidence from a variety of sources such as morphology and vocalisation in a multifaceted approach to species recognition (Crowe *et al.* 1994; Crowe 1999) is essential in obtaining the most robust estimate of evolutionary relationships (Ryan *et al.* 1998).

The issue of species status holds implications for the three taxa that are of conservation concern. The Taita Apalis *A. t. fuscigularis* is listed as Critically Endangered, the Yellow-throated Apalis *A. t. flavigularis* is Endangered and the Namuli *Apalis A. t. lynesi* is Vulnerable (BirdLife International 2000). All three are described as subspecies of *A. thoracica* by Urban *et al.* (1997) but treated as species 'pending resolution of their taxonomy' by BirdLife International (2000). The results of this study however support the distinctiveness of all the lineages that were identified and suggest the presence of hidden diversity in South Africa. The distribution and numbers of the newly identified lineages should be studied in order to assess their conservation status and will be important in terms of biodiversity conservation in particular.

CHAPTER 3

Molecular phylogeny of African *Bradypterus* warblers: discovery of a new putative genus

3.1 Abstract

Phylogenetic hypotheses were constructed for six African members of the genus *Bradypterus* including three southern African endemics. Phylogenetic relationships were inferred from analysis of 603 base pairs (bp) of the mitochondrial DNA (mtDNA) cytochrome *b* gene. Phylogenetic analysis at species level for *Bradypterus* showed that the genus is not monophyletic. One of the species, *B. victorini*, exhibited a level of sequence divergence proportionate to what is found between avian genera (20-24%). Based on phylogenetic distinctiveness, vocalization and habitat choice, *B. victorini* seems to belong to a different genus but without sequencing data for all 10 African and some of the Asian species this issue cannot be clarified. The species status of the other taxa was supported with levels of sequence divergences among them being well within the range found among avian species. The molecular phylogeny largely supported classifications based on habitat and song. The isolated population of *B. barratti priesti* from the eastern highlands of Zimbabwe was almost 2% divergent from *barratti* in South Africa but more than 8% divergent from adjacent populations of *B. lopezi*, confirming that it is closer to *barratti*. However, it is more distinct than other within-species populations sampled, and could be considered as an incipient species. Further sampling of the fragmented populations within the *B. lopezi* complex is likely to reveal additional significant structure. Application of a mtDNA clock calibrated at a rate of 2% sequence divergence per million year (2%/Myr.) suggested that subspecies level splits dated to about 0.1-0.25 Million years ago (Ma) (Pleistocene) whereas species level divergences dated to 4.5-6.5 Ma (Miocene-Pliocene boundary). *B. victorini*, a Fynbos endemic, split from the other taxa much earlier, 16-20 Ma (early Miocene). This is especially noteworthy in the light of growing evidence that the Cape region acted as a repository for ancient animal taxa and holds implications for the conservation status of the region.

3.2 Introduction

Molecular phylogenies provide information that can be valuable at all levels of conservation: It provides insight into the forces that maintain genetic diversity (Balmford *et al.* 1998; Bowen 1999; Crandall *et al.* 2000), assists in identifying populations and taxa under threat or at risk (Avise 1989) and aids in planning of conservation at ecosystem level (Funk *et al.* 2002). Taxonomies based on morphological traits alone have sometimes failed to make adequate phylogenetic distinctions at species and subspecies levels and the value of using molecular techniques in phylogeny reconstruction are well recognised (Avise 1989). In addition, sequencing data have become useful sources of comparative information for phylogenetic inference (Hillis & Moritz 1990) and has led to the development of the field of phylogeography (Avise *et al.* 1987; Avise 1998; Avise 2000). Molecular phylogenies are very useful in resolving species limits and affiliations as part of a multifaceted approach to species recognition (Crowe *et al.* 1994; Crowe 1999) in taxa where more traditional methods such as morphology have struggled to do so (Avise 1989). An example of such a taxon is the genus *Bradypterus*.

There has been wide diversity of treatments in species limits and racial affiliations within *Bradypterus*. The fact that the traditional family Sylviidae, to which *Bradypterus* belongs, is polyphyletic (Sibley & Ahlquist 1990; Goodman *et al.* 1996; Barker *et al.* 2002) complicates matters and makes it difficult to resolve the affinities within the family. As a result, the taxonomy of other members of the family has been questioned (Leisler *et al.* 1997). Currently most authorities recognise 18 species in *Bradypterus*, of which 10 occur in Africa and eight in Asia. However, the Asian species are rather different from the African taxa, and are thought to be better placed in *Locustella* (K Barker pers. comm.). If this proves to be the case, *Bradypterus* would be endemic to Africa. A relatively high proportion of the 10 African species is of conservation concern: two are listed as Vulnerable (Barnes 2000; BridLife International 2000), *B. sylvaticus* and *B. grandis* (Dja River Warbler) and one is classified as Endangered, *B. graueri* (Grauer's Scrub-Warbler; BirdLife International 2000).

Bradypterus warblers are skulking species of dense vegetation and are characterised by little morphological distinction between species (Urban *et al.* 1997). There are thus few morphological characters for characterising species or for inferring phylogenetic relationships. The African species occur in two main habitats: reedbeds and forest understorey (Fig. 3.1). Their songs are well-developed and species-specific, but unfortunately are not well studied. All have fragmented

and restricted ranges with the exception of the Little Rush Warbler (*B. baboecala*), which is widespread in southern and central Africa (Moreau 1966; Urban *et al.* 1997). Several of the forest understorey species are confined to montane forests, and have disjunct distributions similar to those found in many African montane forest birds. Although the origin of these montane species has been the subject of much investigation, most of the hypotheses remain untested (Moreau 1966; Haffer 1969; Diamond & Hamilton 1980; Crowe & Crowe 1982; Roy 1997; Roy *et al.* 1998, 2001; Bowie *et al.* in review).

African *Bradypterus* classifications based partly on habitat but mostly on vocalisations (Dowsett & Forbes-Watson 1993; Urban *et al.* 1997) added to the resolution of previously inferred relationships (Table 3.1). Vocalisations have however, not been able to shed much light on the relationship of *B. victorini* and *B. sylvaticus,* within *Bradypterus* (Urban *et al.* 1997). Molecular studies on other warbler genera have led to some taxonomic rearrangements (Price 1996; Leisler *et al.* 1997; Helbig & Seibold 1999; Irwin *et al.* 2001a). DNA sequence analysis provides a useful tool for investigating evolutionary relationships within as well as between species (Shields & Helm-Bychowski 1988; Quinn 1997). Mitochondrial DNA has been widely used in avian phylogenetic studies (Mindell 1997 and references therein). The cytochrome *b* gene in particular, has been employed in similar studies on other members of the Sylviidae (Helbig *et al.* 1995; Leisler *et al.* 1997, Helbig & Seibold 1999) and also on other avian taxa (e. g. Cooper *et al.* 1992, Helm-Bychowsky & Cracraft 1993, Kornegay *et al.* 1993, Avise *et al.* 1994, Hedges & Sibley 1994). In addition, by applying a molecular clock the branch points in molecular phylogenies can be dated (Brown *et al.* 1979; Shields & Wilson 1987), allowing a crude assessment of whether branching times coincide with geologically dated vicariance events (Bermingham & Avise 1986).

In this manuscript, DNA sequence data from the mitochondrial cytochrome *b* gene were used to assess phylogenetic relationships among six of the 10 African *Bradypterus* Warblers: *B. baboecala*, *B. carpalis*, *B. lopezi*, *B. barratti*, *B. sylvaticus* and *B. victorini,* in particular the placement of the latter two species were assessed. The resulting phylogeny was compared with the current classification outlined by Urban *et al.* (1997) (Table 3.1) in order to assess the level of congruence between phylogenetic relationships based on habitat and song vs. molecular phylogeny.

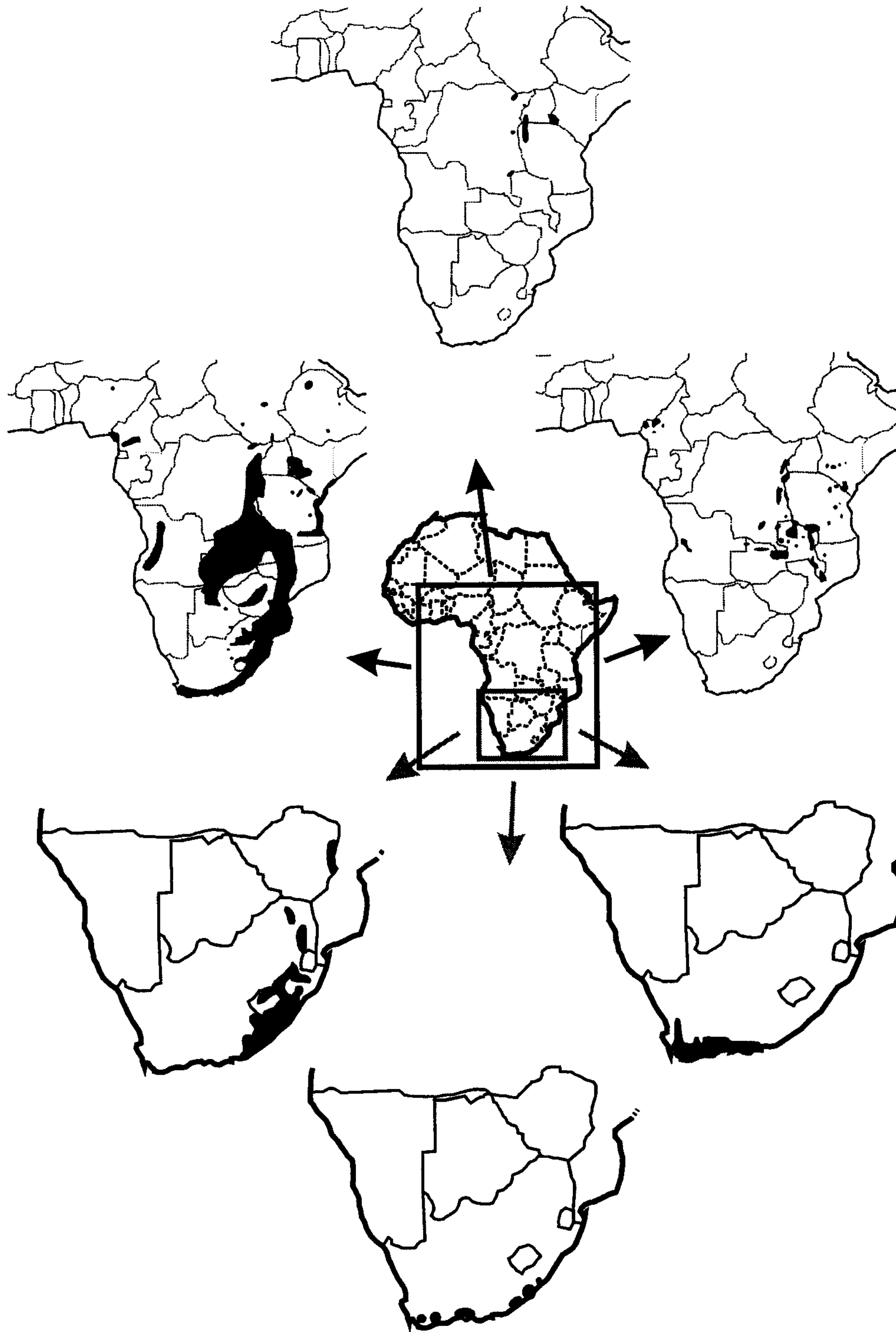

Figure 3.1 Distribution maps of six African *Bradypterus* species: *B. baboecala* (A); *B. carpalis* (B); *B. lopezi* (C); *B. barratti* (D); *B. sylvaticus* (E) and *B. victorini* (F). Maps are modifications of Urban *et al.* 1997 (African distributions) and Clancey 1980 (southern African distributions).

Table 3.1 Classification of African *Bradypterus* warblers based on habitat and song. Modified from Urban *et al.* (1997).

Habitat	Song	Species	Group
reedbeds, swamps bamboo	bouncing ball trill	*baboecala* *carpalis* *graueri* *grandis*	Central African Swamp
wet forest	monotonous chips	*alfredi*	Link between Central African Swamp and Forest & Forest Edge groups
forest edge & interior	more musical trill	*lopezi* *cinnamomeus* *barratti*	Forest & Forest Edge
forest	rapid trill	*sylvaticus*	'Independents' (unknown)
fynbos	rollicking song	*victorini*	

3.3 Materials and methods

Sample collection and DNA extraction

Most samples were obtained from mist-netted birds. The brachial vein was punctured and blood collected with a heparinised capillary tube. It was then stored in buffer (0.1M Tris-HCL, 0.5M EDTA·Na_2, 1.0M NaCl, 0.5% SDS) with a 1:10 ratio of blood to buffer. Additional material was obtained as tissue in DMSO/NaCl (Amos & Hoelzel 1991) or as frozen liver, heart and muscle samples (Appendix 1) from museums and institutional collections.

DNA extraction proceeded as follows: samples (25-50µl of the blood samples or 0.01-0.02 g of the tissue samples minced into small particles) were digested overnight at 55°C with 0.5mg proteinase K (Roche Diagnostics) in 500µl extraction buffer (50mM Tris, pH 7.6, 100mM NaCl, 1M EDTA, pH8.0, 0.5% SDS). Tissue samples occasionally needed an additional 0.5mg proteinase K and an increased incubation to ensure complete digestion. This was followed by RNase A (0.1mg, Roche Diagnostics) treatment for 1 hour incubation at 37°C. Liver samples required increased amounts of RNase A and an extended incubation time. Samples were then extracted three times with phenol and once with chloroform:isoamyl alcohol (24:1). Total DNA was precipitated overnight at –20°C with 0.1 volumes of 3M sodium acetate and 2.5 volumes of 96% ethanol. DNA pellets were collected in a tabletop microcentrifuge at 13000rpm for 30min. This was followed by a 70% EtOH wash whereafter the pellet was collected by spinning at 13000rpm for 30min and resuspended in 50µl Sabax® (Adock Ingram) water preheated to 37°C and then stored at -20°C.

PCR amplifications and sequencing

Approximately 50-100ng of DNA was used as template in a Polymerase Chain Reaction (PCR) (Saiki *et al.* 1988). All PCR reactions were performed in a total reaction volume of 50µl in 200µl thin walled microcentrifuge tubes. In addition to the DNA, the reaction mix contained 2.0 mM $MgCl_2$, 5µl 10 × reaction buffer, 0.2mM of each of the four nucleotides, 25 pmol of each of the two relevant primers (Table 3.2 & 3.3) and 1.5U Super-therm® DNA polymerase (Southern Cross Biotechnology). The primer combinations, concentration of $MgCl_2$ and cycling parameters were adjusted to accommodate species differences (Table 3.3).

The PCR reactions were cycled in a Geneamp® PCR System 9700 (PE Applied Biosystems) under the following conditions: an initial denaturation at 94°C for 5min was followed by: 35 cycles of 30s at 94°C, 30s at the relevant annealing temperature (Table 3.3), 45s at 72°C and a final elongation step at 72°C for 7 min.

Success of PCR reactions were checked on 1.0% agarose (Promega) gels, stained with ethidium bromide, before purification. Products showing specific amplification were purified directly using the High Pure™ PCR Product Purification Kit (Boehringer Mannheim). In the case of products showing non-specific amplification, the different sized bands were separated on a 1.0% agarose gel. A 100bp molecular weight marker was included to identify the correct band, which was

excised from the gel, using a sterile scalpel blade, and purified according to the gel-extraction protocol provided by the manufacturers of the kit. All purifications were eluted in Sabax® water. DNA concentration was estimated spectrophotometrically or by running purifications against DNA of known concentration on ethidium bromide stained agarose gels.

Purified PCR products were sequenced via dideoxy chain termination (Sanger *et al.* 1977), using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Both heavy and light strands were cycle sequenced in a Geneamp® PCR System 9700 with cycling conditions as per the manufacturer's instructions. Cycle sequencing products were precipitated using the NaAc precipitation method (PE Applied Biosystems). Heavy and light strand sequences were imported into Sequence Navigator version 1.0.1 (PE Applied Biosystems), where they were proof read. Consensus sequences were then aligned in Clustal X Version 1.63b (Thompson *et al.* 1997).

Phylogenetic analyses

On the basis of voice and plumage the genus *Cettia* is thought to be the closest relative to *Bradypterus* (Urban *et al.* 1997). Therefore GenBank sequence data for *Cettia cetti* (AJ004798; Helbig & Seibold 1999) as well as three other members of the family Sylviidae, *Acrocephalus aedon* (AF094623; Cibois *et al.* 1999), *Phylloscopus laetus* (AF094624; Cibois *et al.* 1999) and *Sylvia melanocephala* (L77121; Richman unpubl. data) were used to root the phylogenetic trees.

Three methods of phylogenetic analysis were performed in PAUP version 4 (Swofford 1999): neighbour-joining (NJ; Saitou & Nei 1987), maximum parsimony (MP; Hennig 1966) and maximum liklihood (ML, Felsenstein 1981). The best-fit model of DNA substitution as specified by ML was used to determine genetic distances between taxa and to construct a phylogeny using the NJ algorithm. In order to detect saturation, scatterplots of percentage sequence divergence or number of amino acid substitutions versus the number of pairwise transitions and transversions for each of the three codon positions were constructed (Moore & DeFilippis 1997). Saturation was thought to have occurred when DNA substitutions reached an asymptote as percentage sequence divergence increases (Roy 1997).

Table 3.2 Identities and sequences of primers used in this study. General primer names are numbered according to the position of the 3' base in the chicken mitochondrial genome (Desjardins & Morais 1990).

Primer name	Location	General name	Sequence of primer (5' to 3')	Forward/ reverse	Source
L14841	Cytochrome *b*	L14996	CCAACATCTCAGCATGATGAAA	forward	Kocher *et. al* (1989)
H15696	Cytochrome *b*	H15696	AATAGGAAGTATCATTCGGGTTTGATG	reverse	Primer H15547 of Edwards *et. al* (1991)
H15915	Cytochrome *b*	H16064	CATTCTTTGGTTTACAAGAC	reverse	From Pääbo *et al.* (1988)
WBLl1	Cytochrome *b*	L15237	GTCACAGGACTCCTACTAGC	forward	Constructed based on Warbler sequences from GenBank
ND6H	ND6	H16247	GCAGGTTGAGGTTTGTTGTT	reverse	Constructed based on avian mtDNA sequences from GenBank
ND5L	ND5		TACCTAGGRTCHTTCGCCCT	forward	Constructed based on avian mtDNA sequences from GenBank
H522	Control region	H519	GCCTGACCGAGGAACCAGAG	reverse	Modified from Quinn & Wilson (1993)
H1254	Control region	H1253	AGCTTGGCATCTTCAGTGCCA	reverse	Wenink *et. al* (1994)

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Table 3.3 Summary of PCR and sequencing primers used for each of the African *Bradypterus* warblers.

Species	PCR primer combination	PCR (°C) annealing temperature	Sequenced with light strand primer	Sequenced with heavy strand primer
B. baboecala	ND5L & H15696	58	L14841	H15696
B. carpalis	ND5L & H15696	61	ND5L	H15696
B. lopezi	ND5L & H15915	56†	L14841	H15696
B. barratti	ND5L & H15915	53	L14841 or WBLL1	H15696
B. sylvaticus	WBLL1 & H15696	55†	L14841	H15696
B. victorini	WBLL1 & H15696	54	WBLL1	H15696

†non-specific

The MP analyses were conducted under the heuristic search option with all characters unordered and equally weighted with uninformative characters excluded. Three weighted analyses were then conducted: (i) differential weighting for transversions (tv) over transitions (ti), (ii) weighting of codon positions according to the inverse of the variability, as well as a combination of (i) and (ii). Finally, the ML method was used to estimate the following parameters: base frequencies, transition-transversion ratio (ti:tv) and the gamma shape parameter (G) and proportion invariable sites (I). These parameters were thereafter implemented in a heuristic ML phylogenetic analysis using the DNA substitution model identified by ML. The robustness of all the phylogenetic hypotheses was assessed through the bootstrap procedure (Felsenstein 1985), using 1000 random re-samplings. In addition, Bremer support/decay indexes (Bremer 1988; Donoghue *et al.* 1992) were calculated for monophyletic clades using AutoDecay v. 4.0 (Eriksson 1998).

Molecular clock

There is much controversy surrounding the use of a molecular clock and it has been criticised (Hillis & Moritz 1990; Li 1993b; Hedges *et al.* 1996; Ayala 1997; Van Tuinen & Hedges 2001; Rodriguez-Trelles *et al.* 2002). Despite the controversy and problems encountered applying the molecular clock concept, it allows estimation of approximate divergence times (Brown *et al.* 1979; Shields & Wilson 1987) and can be used in formulating preliminary biogeographic hypotheses by assessing whether branching times coincide with geologically dated vicariance events (Bermingham & Avise 1986).

A maximum likelihood ratio test (Felsenstein 1985; Yang *et al.* 1995; Sanderson 1998) was used to test for evolutionary rate heterogeneity, as follows: The likelihood score was estimated in PAUP* on a tree generated with the likelihood analysis using the no branch swapping option, with and without enforcement of a molecular clock (ti/tv ratio, base frequencies, I and G as estimated with likelihood). This resulted in two likelihood scores, the test statistic is equal to twice the ratio of the log likelihood scores, which is chi-square distributed with n-2 d.f., where n is the number of terminal taxa (Yang *et al.* 1995 also see application of test by Hibbett 2001; Piaggio *et al.* 2002). Generally a rate of 2%/Myr. is thought to be appropriate for an array of avian taxonomic orders (Shield & Wilson 1987; Tarr & Fleischer 1993; Fleischer *et al.* 1998; Klicka & Zink 1999), although this rate is thought to be too low for passerines with short generation times (Mooers & Harvey 1994; Leisler *et al.* 1997). In addition when saturation occurs, estimates of splitting event times based on overall divergence may underestimate the real date. Therefore, it may be more appropriate to use the calibration of Irwin *et al.* (1991) of 0.5%

third codon position transversion (tv) divergence per Myr. (0.5% third position tv/Myr.) when saturation is discovered. This calibration is based on the discovery of a linear relationship between transversions in cytochrome *b* sequences and divergence dates of different taxa of ungulate mammals for which there is a good fossil record (Irwin *et al.* 1991).

3.4 Results

Sequence variation

Six hundred and three base pairs of cytochrome *b* (Appendix VI) were analyzed for 12 haplotypes in *Bradypterus* and four outgroups. The sequences of the following individuals were identical and therefore only one of the sequences was included in further analyses: all three *B. victorini* individuals and both *B. lopezi* individuals. Maximum-likelihood estimates of base frequencies showed a bias toward C (36.7%) followed by A and T (30.0% and 22.5%, respectively), with G having the lowest frequency (10.9%). Of the 603 bp sequenced, 225 sites were variable (162 within *Bradypterus* alone) (Table 3.4), 155 were parsimony informative and I was 0.460. The largest proportion of variable characters was located in the third codon position (167) followed by first (43) and second codon positions (15). Transitions outnumbered transversions 4:1 and G was estimated 0.671. Maximum-likelihood identified the HKY85 model with I and G as the model of evolution that best fit the data.

Table 3.5 is a summary of the HKY85 and HKY85+I+G pairwise distances among haplotypes. Excluding within species comparisons of *B baboecala* and *B sylvaticus*, the HKY85+I+G distances were larger than the HKY85 distances especially for pairwise comparisons between the ingroup and outgroup taxa (including *B. victorini*) as well as among the outgroups. The sequence divergence (HKY85) found between *B. victorini* and the other African *Bradypterus*, 18.0-19.6% (average of 18.6%) was on the same level as what was found between the outgroups and *B. victorini* as well as the other African *Bradypterus:* 15.5-19.1% (average of 17.9%) and 16.2-23.5% (average of 19.4%), respectively. The sequence divergence among species *baboecala*, *carpalis*, *lopezi* and *barratti* ranged from 9.1-11.6% (average of 10.7%), excluding *B. barratti* 1 from Howick. *Bradypterus sylvaticus* was slightly more divergent, 11.0-12.9% with an average of 11.7%. The intraspecific sequence divergences were as follow: 0.2–0.5% within *B. baboecala,* 0.3 *within B. sylvaticus,* 0% within *B. victorini,* 0% within *B. lopezi* and 1.9–12.2% within *B. barratti.* The large divergence within *B. barratti* apparently is a result of hybridisation; one of the

Table 3.4 Aligned nucleotide sequences of the variable positions in the mtDNA cytochrome *b* gene of twelve *Bradypterus* taxa plus outgroups: *Sylvia melanocephala*, *Cettia cetti*, *Acrocephalus aedon* and *Phylloscopus laetus*. Dots indicate identity with first line of each block. Unknown nucleotides are marked by '?'.

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                                                    1111111111111111111111111111111111111111112222222222222222222222222222222222333333
                         11112222222333334445556666677888889999000011122222334444455566677778888888999000111122233444555666777788999000011
Taxon/Node               134692358124789034792581570367928246780367258914706792801457069568145703467892581470369025140392581480369581470139 34
-------------------------------------------------------------------------------------------------------------------------------------------
tongensis 1              GCCACCTACAGTTCTACCACCACTCTCACTATCGCCCCACCACCCTCCCCGACCCCACCACACTGCATACTACCGCAAAATTCACCATACAAACGTCGCACACAACCTCACCAAAT
tongensis 2              .....................................................................................................................
transvaalensis 1         .....................................................................................................................
transvaalensis 2         .....................................................................................................................
carpalis                 ???????????????????????????..C.C.A.T..C..........GA...TGG........T..G....A.............C.....TA.AA.G.....ATC........
sylvaticus 1             ....T....C.CCA......A.TC....TC.C......C...TT......A............CAT....C..AA....TC...T..C.........A.........C...T....
sylvaticus 2             ....T....C.CCA........TC....TC.C......C...TT......A............CAT....C..AA....TC...T..C.........A.........C...T....
lopezi                   .........T.CCA.C......T....G.C.C......C..G........A..T..T..G.G.C.......C.AAT....C......CG.T......AT......A...G......
barratti 1               ....T....C.CCA......A.TC....TC.C......C...TT......A............CAT....C..AA....TC...T..C.........A.........C...T....
barratti 2               .........T.CCA.G..G....C.C.G.C.C......C.........T.A..T......T..C...C...T.GTT...........C.TC................C...T....
barratti 3               .........T.CCA.G.......C.C.G.C.C......C.........T.A..T......T......C...T.GTT...........C.TC............T..C...T.G..
victorini                T...T.GT.CTCCA.C..G.A..CTA...C.C....AA.....T.A..TAAT..T.......ACAT.A..CT.ACTTC...C...T.CT.....A.AC.C..A..A....A...GC
Sylvia                   TATG...CT..CCA....G...TC.A...CTC..T.ATC......AT.T.A..........G...A....G..AA.CCGC....T.TCCA.G..A.AAT....TTA.CT.A...GC
Acrocepalus              TT..A.AC.C.C.G..TGG.AGTATA.C.CT......TC.....T...T.AT..A.C......CA.C....G.A..CCGC.C..ATCCGA....A.AAT..C.TTA....A.G.GC
Cisticola                TT.....C.C..CA....G....A.A.C.C.CT....TC.T....CAT..T...........GC.A.G..A..TA.C...C...T.CCC.......AC?.T...C...A.AT..GC
Phylloscopus             T...A.ATTC.CCAC.....T..C.AT..CCC.....TT.T......T..AT..A.GT.....CA.....A.TAA..C.C.C.CG..C..G....CAAT......A....A...GC
Cettia                   TT...TGC.CACCA.G...T..T..A..TC....T..TCT..........CCAT..T.T.....A..A.TC.TA.......CT.TT.CCA..C...AA.C.....A..T....GC.
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Table 3.4 (continued)

Taxon/Node	3333333333333333333333333333333444444444444444444444444444444444444445555555555555555555555555555555555555555555566 1222333344445555666667788899999000011111222233334444455556667788899990000111122222333333344444445556666777778899900 5147036925681467034695814502679235812478036902581478934692581767926890147016913579123456702345672590148013692814703
tongensis 1	AAACAACCCATATATCCCACCTCACGTCACAACACCCCCCTACAAACCCCTCTCACCGCTCCCCACTCACAATCCGCCCCCCTATCCTAGTCAGCTACCTACTCCGCCGTCCACC
tongensis 2	.G...
transvaalensis 1	.G..T....
transvaalensis 2	.G...A..................................T....
carpalis	.........C.C...T...TCT...C.G..G..................T...A...AT.......A.....C..A......C.....GA.....C............AC...??
sylvaticus 1	.C......TGCCCC..TTC..C.........C......A..C....A...C......ATC......AT...GC..AT..T..C.C...CA..........G.C..AT.AC.....
sylvaticus 2	.C..G...TGCCCC..TTC..C.........C......A..C....A...C......ATC......AT...GC..AT..T..C.C...CA..........G.C..AT.AC.....
lopezi	.......TA.C.C........C............T......G.......TC.....TA.CTT..........CT.A...A..C......A.AG..C......CT.ATTAC...T.
barratti 1	.C......TGCCCC..TTC..C.........C......A..C....A...C......ATC......AT...GC..AT..T..C.C....A..........GTC..AT.AC...??
barratti 2	.T.....T..C...........A...C.G.G..................TC.....TATCT....TA.....C..A......C.C....A.AG..CGT...TCT.A..AC...T.
barratti 3	.C.....T..C...........A...C.G.G.................T.C.....TATCT....TA.....C..A......C..T...A.AG..CGT...TCG.A..AC...TT
victorini	.T......ACC........T.CA.T....GC....T.A.TC.T...AA..AACAGG.A.C.....TA....GC..A...ATA..C....T..TC.CCATCCTAG.AA.AC.A...
Sylvia	C.C.G.....C.C....TCT.C.C....C...T.AT....A...T.GT..C......ATC....C..........CT..TTTC....CTCC.TC.C.AGCCTC..AA.AC.GC..
Acrocepalus	..CT....ACC.C...T....C...AC.GTCT.....A..C.TT..AA...T.A.TAATC....T..AGT..CTTATT..T.C.C...CCCTGCTCCAGCCTCATAA.AC.A...
Cisticola	.T....TTACCCC.C.TT...CT.T........C..TA..CC....AA..AA.A..AA.....AT.......CT.A...AT.C.....TACT...C.ATCTTC..AT.ACTA...
Phylloscopus	.C.......CC.......C......A..G.CC..G..A..A.....AA..C..A.TAA.C....C..T....CT.C....T.A.....CTC.TC.CCATC.TCA.AA.AC.GG..
Cettia	.C...TT.ACC.C..T...TTAT...CT..C.......ATC....GTT..CA.A.TAA.C..T..T.T.TT.C.TAT.T.T..G..TCTC.TGC.CCATCC.CGTAA.AC.A..T

Table 3.5: Percentage sequence divergence including all substitutions among six African *Bradypterus* and five outgroups in the cytochrome *b* gene. Above the diagonal are figures for HKY85 including the proportion of invariable sites (I) and the gamma distribution parameter (G) and below the diagonal are HKY85 distances.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*B.b. transvaalensis 1*	-	0.2	0.2	0.3	18.8	17.1	17.2	17.4	18.5	18.6	13.2	36.7	55.8	48.8	30.8	37.1
2	*B.b. transvaalensis 2*	0.2	-	0.3	0.5	18.3	16.6	16.7	16.9	18.0	18.1	12.8	35.9	54.4	47.6	30.8	37.2
3	*B.b. tongensis 1*	0.2	0.3	-	0.2	18.3	16.6	16.7	16.9	18.0	18.1	12.8	35.9	54.4	47.5	30.1	36.2
4	*B.b. tongensis 2*	0.3	0.5	0.2	-	18.3	16.6	16.8	16.5	18.0	18.1	12.3	35.9	53.0	47.6	30.1	35.4
5	*B. barratti 1*	12.0	11.8	11.8	11.8	-	18.0	19.2	16.3	0.3	0.7	16.9	29.8	44.7	43.8	27.8	29.3
6	*B. barratti 2*	11.2	11.0	11.0	11.0	11.6	-	2.0	11.3	19.2	19.4	16.3	29.2	45.5	48.3	31.4	36.7
7	*B. barratti 3*	11.3	11.1	11.1	11.1	12.2	1.9	-	13.4	21.0	21.2	17.6	34.3	52.3	42.1	32.8	37.5
8	*B. lopezi*	11.3	11.1	11.1	10.9	11.1	8.4	9.5	-	16.4	16.5	18.0	37.7	46.1	46.0	29.7	33.6
9	*B. sylvaticus 1*	11.9	11.7	11.7	11.7	0.3	12.1	12.9	11.2	-	0.3	16.3	30.6	44.3	43.4	27.6	29.7
10	*B. sylvaticus 2*	11.9	11.7	11.7	11.7	0.7	12.1	12.9	11.2	0.3	-	16.9	31.8	46.2	43.8	28.4	28.5
11	*B. carpalis*	9.3	9.1	9.1	8.9	11.3	10.9	11.5	11.6	11.0	11.3	-	33.3	38.7	46.0	30.6	32.7
12	*B. victorini*	19.5	19.3	19.3	19.3	17.5	17.2	18.7	19.6	17.8	18.2	18.2	-	31.2	37.9	25.0	35.4
13	*Acrocephalus*	23.5	23.3	23.3	23.0	21.4	21.8	23.0	21.6	21.2	21.6	19.7	17.8	-	43.0	26.4	33.4
14	*Cettia*	21.4	21.2	21.2	21.2	20.6	21.4	20.2	20.7	20.4	20.4	20.6	19.1	20.2	-	42.5	47.8
15	*Phylloscopus*	17.4	17.4	17.2	17.2	16.3	17.6	18.1	17.2	16.2	16.4	17.1	15.5	15.3	19.9	-	28.0
16	*Sylvia*	19.2	19.2	19.0	18.8	17.1	19.4	19.6	18.5	17.2	16.8	18.0	19.3	18.1	21.9	16.2	-

barratti samples was only 0.3-0.7% divergent from *B. sylvaticus*. These taxa meet in southern Kwa-Zulu Natal and the close similarity between these samples suggested that hybridisation may take place at least occasionally. The distance between the other South African *barratti* sample (Weza) and the isolated population from eastern Zimbabwe (1.9%), was considerably greater than other intra-specific distances, emphasising the distinctness of this population.

The number of amino acid substitutions per pairwise comparison is shown in Table 3.6. There were no amino acid substitutions among subspecies of *B. baboecala* or *B. sylvaticus*. Within *B barratti* however, there were two amino acid substitutions between *B. barratti godfreyi* (Weza) and *B. barratti priesti* (Vumba) and five between *B. barratti godfreyi* (Howick) and the other two individuals. Within *Bradypterus* there were five to nine amino acid substitutions as opposed to the 19-22 between *B. victorini* and *Bradypterus*, 12-20 between the outgroups and *Bradypterus* and 19-21 *between B. victorini* and the outgroups. Pairwise amino acid substitution comparisons exhibited a leveling off in third position comparisons corresponding to the saturation found in sequence divergence for the same codon position (Fig. 3.2). The saturation plots (Fig. 3.2) reached a transition asymptote for third position transitions after 15% sequence divergence and five amino acid substitutions. These saturated data points all occurred between *B. victorini* and the other *Bradypterus* warblers. Transversions at the third position did not appear to be saturated (Fig. 3.2).

Phylogenetic analyses

Although three separate phylogenetic analyses (NJ, MP & ML) were conducted, the tree topologies were in agreement. The correlation between the trees was considered to be an indication that the base composition bias (Collins *et al.* 1994; Lockhart *et al.* 1994; Swofford 1998) towards C did not influence the results. Both Maximum likelihood and Modeltest identified HKY85 including I and G as the model of evolution that best fit the data. The MP analysis with and without down-weighting codon positions according to the inverse of their variability (position 1 = 5; position 2 = 15; and position 3 = 1) resulted in the same tree topology (Fig. 3.3; only tree without weighting is shown). The MP analysis with down-weighting for both ti:tv ratio (4:1 as estimated by ML) alone and in combination with weighting for variability as well as the ML analysis resulted in less resolved trees (results not shown).

Table 3.6 Pairwise comparisons among six African *Bradypterus* species and four outgroups for the cytochrome *b* gene. Above the diagonal are uncorrected P distance values (in percentage sequence divergence) for third position transversions alone and below the diagonal are number of amino acid changes.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*B.b. transvaalensis 1*		0.0	0.0	0.0	6.0	4.0	5.0	5.5	6.5	6.0	3.4	19.4	22.9	16.4	18.4	17.9
2	*B.b. transvaalensis 2*	0		0.0	0.0	6.0	4.0	5.0	5.5	6.5	6.0	3.4	19.4	22.9	16.4	18.4	17.9
3	*B.b. tongensis 1*	0	0		0.0	6.0	4.0	5.0	5.5	6.5	6.0	3.4	19.4	22.9	16.4	18.4	17.9
4	*B.b. tongensis 2*	0	0	0		6.0	4.0	5.0	5.5	6.5	6.0	3.4	19.4	22.9	16.4	18.4	17.9
5	*B. barratti 1*	7	7	7	7		6.0	7.0	8.5	0.5	1.0	6.7	20.5	22.1	17.5	17.5	20.1
6	*B. barratti 2*	7	7	7	7	5		1.0	3.5	6.5	6.0	5.1	18.4	23.9	16.4	18.4	19.9
7	*B. barratti 3*	5	5	5	5	5	2		4.5	7.5	7.0	6.2	18.4	22.9	16.4	18.4	19.9
8	*B. lopezi*	5	5	5	5	8	6	4		9.0	8.5	5.1	17.9	22.4	15.9	19.9	20.4
9	*B. sylvaticus 1*	7	7	7	7	0	5	5	8		0.5	7.3	20.9	21.4	16.9	16.9	19.4
10	*B. sylvaticus 2*	7	7	7	7	0	5	5	8	0		7.4	21.4	21.9	16.4	16.4	18.9
11	*B. carpalis*	5	5	5	5	9	8	7	9	9	9		16.4	20.1	14.5	16.0	17.4
12	*B. victorini*	22	22	22	22	20	19	21	23	20	20	20		18.4	14.9	16.9	21.4
13	*Acrocephalus*	20	20	20	20	20	19	21	21	20	20	20	22		17.4	11.4	16.9
14	*Cettia*	19	19	19	19	19	20	19	20	19	19	20	21	20		15.9	20.4
15	*Phylloscopus*	14	14	14	14	12	17	15	15	12	12	16	19	12	17		15.4
16	*Sylvia*	15	15	15	15	14	15	15	16	14	14	17	21	18	21	11	

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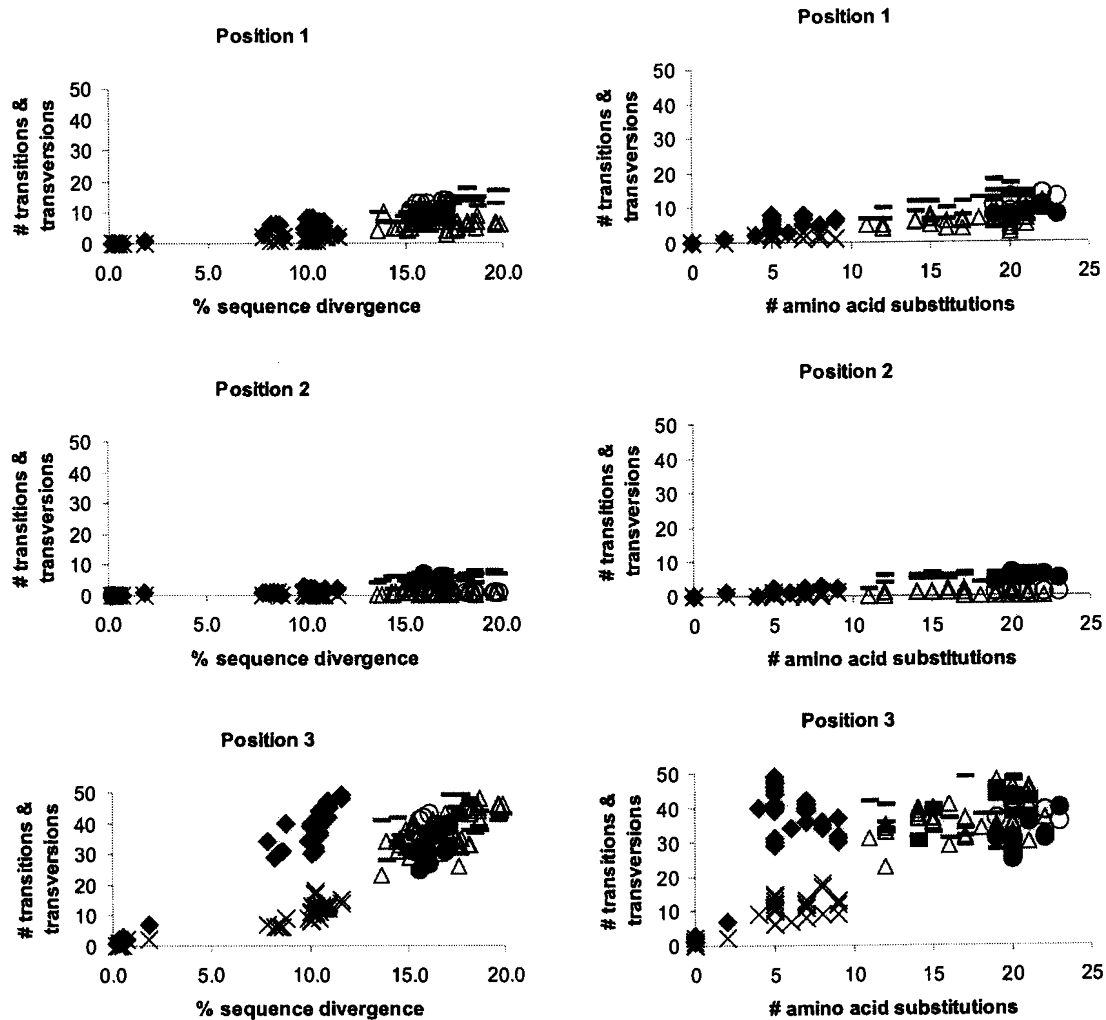

Figure 3.2 Saturation curves of first, second and third position transitions and transversions versus: % sequence divergence (A, B, C); and amino acid substitutions (D, E, F) per pairwise comparison of African *Bradypterus* warblers for cytochrome *b*. Transitions and transversions of pairwise comparisons within *Bradypterus* excluding *B. victorini* are shown in closed diamonds and crosses respectively. Transitions and transversions of pairwise comparisons between *B. victorini* and the other *Bradypterus* warblers are indicated by closed and open circles respectively. Transitions and transversions of pairwise comparisons among the outgroups as well as between the outgroups and *Bradypterus* including *B. victorini* are represented by horizontal lines and open triangles respectively.

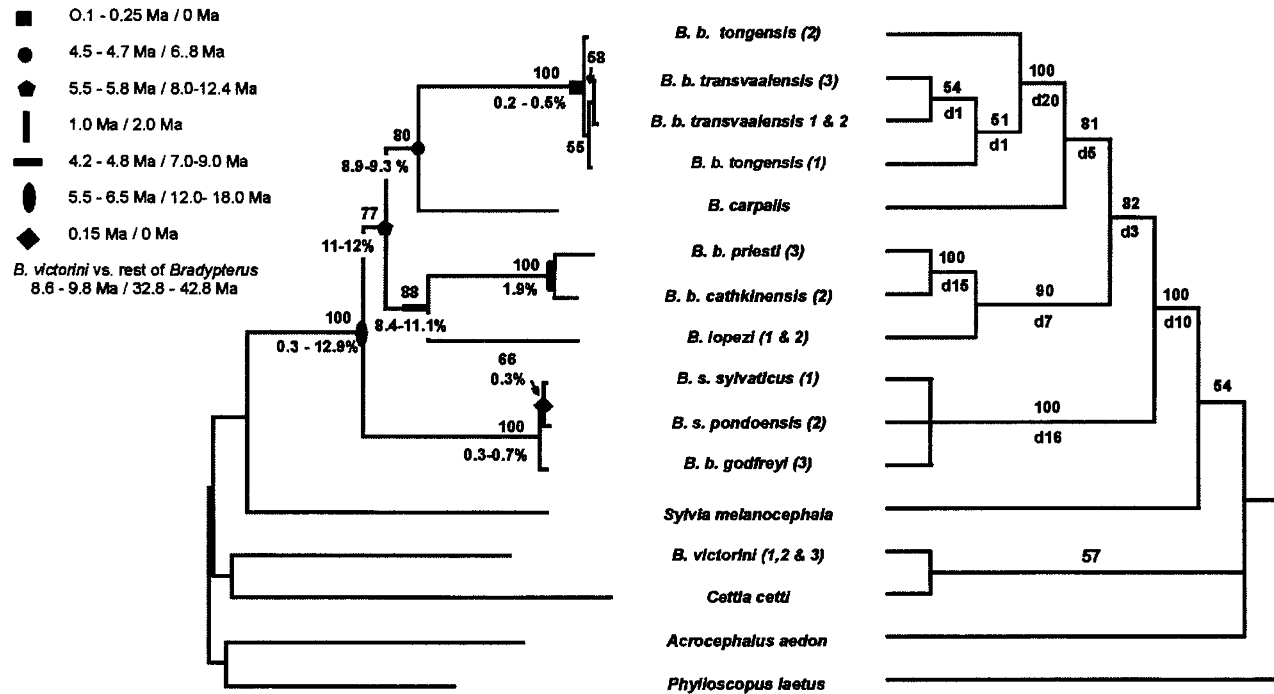

Figure 3.3 Reconstruction of the molecular phylogeny of six African *Bradypterus* warblers based on cytochrome *b* sequences (603bp) and using four other sylviid warblers (*Sylvia*, *Acrocephalus*, *Phylloscopus* and *Cettia*) as outgroups. The neighbour-joining phylogram is shown on the left, with bootstrap values (1000 replicates) at nodes and HKY 85 distances shown below the tree branches. The maximum parsimony cladogram with bootstrap values (1000 replicates) indicated at nodes, is shown to the right. Bremer support or decay index values for the branching nodes are indicated below the tree branches. Estimated times of divergence and corresponding symbols are indicated in the figure legend. Estimated time of divergence by assuming a molecular clock of 2% per Myr. is shown first followed by estimating time of divergence by using 0.5% sequence divergence per Myr. for 3rd position tv.

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Five phylogenetic results are well supported: First, although *Sylvia*, *Acrocephalus*, *Phylloscopus* and *Cettia* were specified as outgroups in each of the phylogenetic analyses, all three analyses (NJ, MP and ML) placed one of the ingroup taxa, *B. victorini*, together with the outgroups as basal to the remaining ingroup taxa (Fig. 3.3). Consequently the monophyly of *Bradypterus* was not supported by any of the analyses (Fig. 3.3). For ease of reference in the remainder of the manuscript *Bradypterus* will be referred to as excluding *B. victorini*. Secondly, the exact relationships between the outgroup taxa and *Bradypterus*, as well as among the outgroup taxa could not be resolved. Thirdly, there was high bootstrap support for a monophyletic clade consisting of species *baboecala*, *carpalis*, *barratti* and *lopezi* a monophyletic clade. Of these the first two and the second two grouped into two separate monophyletic clades. The *B. baboecala* subspecies complex formed a monophyletic group. Fourthly, there was also very high bootstrap support for a larger monophyletic clade, which included the basal grouping *B. sylvaticus* in addition to the previously mentioned species. Lastly, *B. barratti* seemed to be paraphyletic although this is likely to be a result of introgression.

Molecular clock

The difference in likelihood scores was not significant according to the likelihood ratio test (χ^2 = 19.8, d.f. = 14) and the molecular clock was not rejected. A molecular clock was subsequently used to infer estimates of relative divergence dates (Fig. 3.3). The estimated times of divergence based on a molecular clock of 2%/Myr. for total substitutions (HKY85), dated subspecies divergences to the Pleistocene (0.1-0.2 Ma), species level divergences to the Pliocene-Miocene boundary (4.2-6.5 Ma) and the split between *B. victorini* and the remaining *Bradypterus* warblers to the Miocene (average of 9.3 Ma). However, given that the saturation plots (Fig. 3.2) showed evidence of saturation in pairwise comparisons between *B. victorini* and the *Bradypterus* warblers, calculations of diversification times based on overall divergence may underestimate these particular splitting events. A molecular clock of 0.5% third position tv /Myr. was therefore also applied to date splitting events. This method dated splitting events to much earlier than estimations based on calibration of 2%/Myr. (Fig. 3.3). The difference in estimated times of divergence between the two alternative methods are shown in the legend of Fig. 3.3 where dating based on a 2%/Myr. calibration is followed by estimations based on 0.5% third position tv/Myr. Species level divergences were dated to the Plio-Miocene boundary and the Miocene (4.0-7.3 Ma) and the split between *B. victorini* and the remaining *Bradypterus* warblers to the Oligocene (average of 37.4 Ma).

3.5 Discussion

Phylogeny

Although *B. sylvaticus* was slightly more distantly related to the other *Bradypterus* warblers the level of sequence divergences among the *Bradypterus* species, (8.9 to 12.9%) were well within the range found among congeneric avian species (1.5 – 13.5%; Johns & Avise 1998). In all cases where more than one individual was sequenced per species, the sequences were either identical (apart from the divergent *B. barratti priesti* sample from Vumba, Zimbabwe) or displayed reciprocal monophyly. The molecular phylogeny and levels of sequence divergence between species therefore indicated that the species are distinct evolutionary lineages and thus supported their specific status based on the current dataset. It would however be more insightful to include the missing four African and some of the Asian *Bradypterus* species as well as a broader outgroup selection. The basal position of *B. sylvaticus* within *Bradypterus* suggest that African *Bradypterus* is primarily a forest species that probably subsequently radiated into swampy habitats.

The paraphyly of *B. barratti* could possibly be the result of past and/or ongoing hybridisation between *B. barratti* and *B. sylvaticus* rather than mistaken identification by the collector. The five amino acid substitutions between *B. barratti godfreyi* (Howick) and the other two *barratti* individuals suggested that the Howick sample didn't have *B barratti* mtDNA. Since mtDNA is maternally inherited it is possible that hybridisation resulted in offspring with mtDNA from one parent (*B. sylvaticus*) and nuclear DNA from the other (*B. barratti*) and would explain why the individual might resemble one species but have mtDNA from another. This possibility needs to be tested by sampling more *B. b. godfreyi*. However, the collection location of the divergent sample (*B. barratti godfreyi* (Howick) is quite distant from the nearest known *B. sylvaticus* population – ca 140 km – and also at considerably higher elevation (the two are altitudinally segregated in their area of overlap), whereas the other *barratti* sample from KwaZulu-Natal was collected only 45 km from the Oribi population of *B sylvaticus*). This suggests that hybridisation between *B. barratti* and *B. sylvaticus* is possibly not the most likely explanation for the divergent *B. barratti godfreyi* sample.

Probably the most fascinating result is that *B. victorini* does not seem to be a *Bradypterus* warbler. On the basis of sequence divergence, *B. victorini* is as different from any of the other *Bradypterus* warblers (in this study) as both *B. victorini* and the other *Bradypterus* warblers are to the outgroup taxa. The sequence divergence also falls well outside the range found for the

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cytochrome *b* gene among congeneric species (average 6.5%, ranges from 1.5 to 16%; Johns & Avise 1998) and is more in agreement with distance between confamilial genera in birds (average 13%, ranges from 12% to 23%; Johns & Avise 1998). The distinctness between *B. victorini* and the *Bradypterus* warblers is reflected in the distribution of the number of amino acid changes (Fig. 3.2) as well as the fact that saturation only seemed to have occurred in pairwise comparisons between *B. victorini* and the *Bradypterus* warblers (Fig. 3.2).

Bradypterus victorini is not only very distinct on a molecular level, its distinctive song is completely different to a typical *Bradypterus* song (Fig. 3.4). The typical *Bradypterus* song starts with single introductory notes that increase in frequency, ending in a trill. When comparing sonograms of *Bradypterus* species *B. sylvaticus, B. barratti* and *B. baboecala* (Fig. 3.4 A, B and C) with a sonogram of the vocalization of *B. victorini* (Fig. 3.4 D) the difference is obvious. Also in *B. victorini* both males and females sing whereas in the rest of *Bradypterus* only males sing (P.G. Ryan pers. comm.). Therefore based on its phylogenetic uniqueness, its distinctive song that is completely different to other *Bradypterus* songs and the fact that it is the only one species that is restricted to Fynbos, Victorin's warbler, *B. victorini*, does not seem to be a *Bradypterus* warbler.

Although parsimony analysis of cytochrome *b* sequences grouped *B. victorini* with *Cettia*, there was no bootstrap support for this relationship and no close relatives of *B. victorini* were identified. The exact placement of *B. victorini* within the family Sylviidae and its relationship to the genus *Bradypterus*, therefore, remains unclear. Helbig & Seibold (1999) could not identify close relatives of the reed warbler group using cytochrome *b*. They found that cytochrome *b* resolved phylogenetically recent relationships quite well but more ancient nodes remained poorly resolved. It was suggested by Leisler *et al.* (1997) that this is caused by multiple substitutions resulting in homoplasy. If there are too many homoplasies, "wrong" results with little support may be generated. Use of a more conservative molecular marker such as the 16S rRNA or RAG1 (Barker *et al.* 2002, Johansson *et al.* 2002) may overcome these problems.

Molecular phylogeny vs. habitat & song classification

In general there was concordance between classification based of habitat and song vs. the molecular phylogeny. The members of the Central African swamp group (*B. baboecala* and *B. grandis*) grouped together on a molecular level as did the members of the Forest and Forest Edge group (*B. barratti* and *B. lopezi*). Classification based on the habitat/song placed *B. sylvaticus* and *B. victorini* at the end of the *Bradypterus* list as 'independents' (assuming 'independent' means

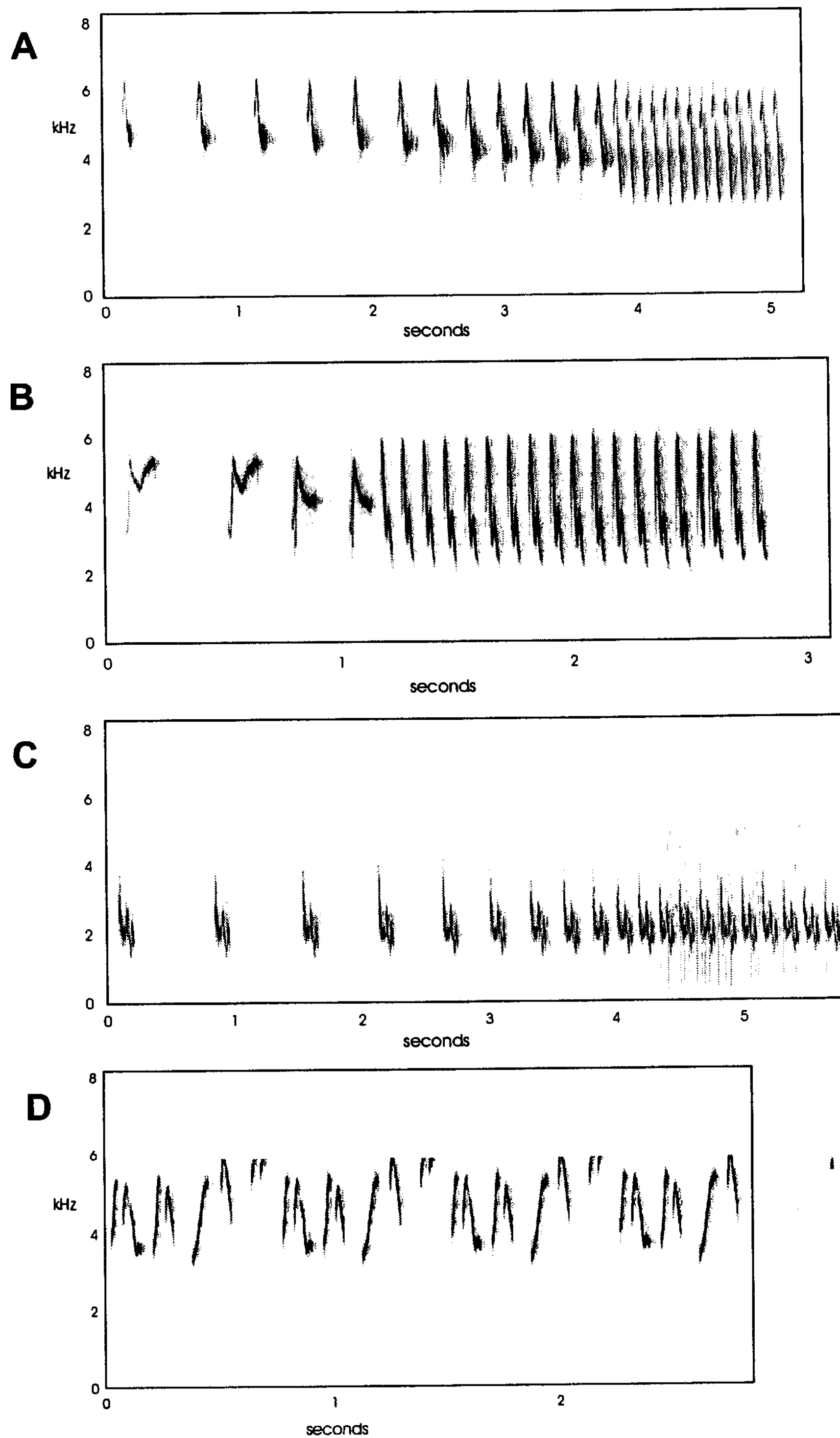

Figure 3.4 Sonograms (Canary 1.2; PG Ryan pers. comm.) of the songs of *Bradypterus sylvaticus* (A); *B. barratti* (B); *B. baboecala* (C) and *B. victorini* (D).

that their affiliation to the other members of the genus is uncertain; Urban *et al.* 1997). The dubious affiliation of *B. sylvaticus* and *B. victorini* were supported by the molecular data, although it is unclear from Urban *et al.* (1997) why *sylvaticus* wasn't included in the Forest and Forest Edge group. Based on sequence divergence *B. sylvaticus* was slightly more distantly related to the other *Bradypterus* Warblers than what those were to each other. It did however fall in a monophyletic group with the other *Bradypterus* warblers and the level of sequence divergence separating it from the other members of the genus was within the range found between congeneric species and well below what was found between confamilial genera (Johns & Avise 1998). This suggests that it is a member of the genus *Bradypterus* whereas *B. victorini* does not seem to be.

Molecular clock and biogeographic hypotheses

The estimated Pleistocene divergences for subspecies and Miocene-Pliocene (clock of 2% per My) up to Miocene (clock of 0.5% for 3rd position tv) divergences for species is congruent with Miocene species level divergences in *Phylloscopus* warblers (5.5-7.0Ma; Helbig *et al.* 1995). The third position tv dating scheme placed the split between *B. victorini* and the remaining *Bradypterus* in the Oligocene, which predates the 2%/Myr. dating scheme estimation by 28.1 Myr. and is older than Sibley & Ahlquist's (1990) estimate for *Phylloscopus* warblers (19 Ma). As a result of saturation at third position transitions (Fig. 3.2), the estimations based on 2%/Myr. seems to have underestimated the divergence times in particular for the split between *B. victorini* and the remaining *Bradypterus* species. The Oligocene split between *B. victorini* and the other *Bradypterus* warblers should however not be treated as an absolute dating. It should only be regarded as an indication that Victorin's Warbler is an old taxon and possibly of a pre-Pliocene age.

The basal position of *B. sylvaticus* (Western and Eastern Cape endemic) within six of the 10 African *Bradypterus* species as well as the phylogenetic uniqueness and relictual nature of Victorin's Warbler, is significant in the light of growing evidence that the Cape acted as a repository for ancient animal taxa. Ancient phylogenetic lineages or relictual taxa within the Cape are known for plants (seven endemic families; Cowling *et al.* 1992; Cowling & Richardson 1995) and for vertebrates (13 Gondwana relicts in 21 Cape Peninsula endemics; Picker & Samways 1996; Sharratt *et al.* 2000). Very few data are available for vertebrates, however, although

Wieczoreck *et al.* (2000) showed the basal position of the Cape endemic *H. horstocki* within the frog genus *Hyperolius*. The most compelling evidence for birds is that the sugarbirds (Cape Sugerbird, *Promerops cafer* and Gurney's Sugerbird, *Promerops gurneyi*) is an endemic familiy (although extending up to eastern Zambia) and rockjumpers (genus *Chaetops*) are extremely primitive within the Old World oscine passerine radiation, and probably warrant family-level status (reported at the IOC; Anon 2003). Impson *et al.* (1999) mentioned 16 endemic freshwater fish in the Cape Floristic Kingdom. The current study therefore provides further evidence of Fynbos antiquity. Adding another relict to this region would add further to the taxonomic uniqueness of the Fynbos, which is an important consideration in setting conservation priorities (ICBP 1992) of a region that is already listed as having a high biodiversity conservation status as one of the worlds endemic bird areas (Stattersfield *et al.* 1998). In general conservation of the Cape Fynbos is important for the preservation of biodiversity and may serve as a natural laboratory where the evolutionary processes of adaptation and speciation can be studied as concluded by Howarth (1983).

The genus *Bradypterus* is not only important in terms of the conservation of the species within the genus. More broadly, making a phylogenetic and phylogeographic comparison between the genus *Bradypterus* and other similarly distributed taxa could help identify areas of concordant patterns of species richness and endemism that are genetically and phylogenetically unique for the purpose of conservation. In addition by including the *B. lopezi* complex and *B. cinnamomeus* as models, hypotheses on the origin of the disjunctions seen in many Afromontane birds could be tested, provided that an adequate number of samples can be obtained.

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CHAPTER 4

Conclusions

Presented in this thesis are the results from two phylogenetic studies on montane birds in Africa. Due to their overlapping distributions they are ideal subjects for a comparative phylogeography study. Such a study would allow the inference of common patterns of vicariance and hence the formulation of biogeographic hypotheses that unifies the historical patterns of change in Afromontane forests and speciation in montane birds. Unfortunately an inadequate number of samples as well as uneven geographic sampling prevented the attainment of this goal. In both studies the molecular data have shed new light on phylogenetic relationships previously based on morphology. The results of both studies indicate that the Cape region is unique and old in terms of its montane-associated avifauna. In addition some interesting and significant patterns were found for both studies separately that warrants further investigation.

Twenty-one subspecies are recognised within the *A. thoracica* complex of which three are of conservation concern due to their restricted ranges and or unique plumage. There was however molecular support for only 11 historically isolated clades. Due to the paucity of samples in some regions, as well as the absence of sequencing data for two of the traditionally recognised subspecies, some regions may contain some additional diversity that might be discovered when the gaps in the sampling are filled in. In many instances the sequence divergence among these clades were on the same level as what has been found among other avian species. All 13 clades would be recognisable under the Phylogenetic Species concept although a more multifaceted approach to species recognition that includes vocalisation data is recommended. A lower level of genetic diversity was expected among the contiguously distributed subspecies in southern Africa versus the isolated ones in East Africa, but in stead there were some unexpected deep phylogeographic breaks within South Africa that warrants further investigation.

Presented and discussed in this thesis are results which indicate that Victorin's Warbler *B. victorini* possibly is not an African *Bradypterus* warblers. Two lines of evidence suggest that *B. victorini* is highly distinct from the other African *Bradypterus*. In the first place it formed a distinct evolutionary lineage with levels of sequence divergence that fall in the range found among not only sylviid genera (Chapter 3), but other avian genera as well (Johns & Avise 1998). Secondly, although extensive vocalisation studies and analyses were not performed in this study, preliminary analyses indicate that the song of *B. victorini* is highly distinct from that of other African *Bradypterus* warblers. In addition, uncharacteristic of *Bradypterus* warblers where only males sing, both *B. victorini* males and females sing. The discovery of very distinct and old species, endemic to the Cape Floristic Region is especially noteworthy in the light of growing evidence that the Cape region has acted as a repository for ancient animal and plant taxa and holds implications for the conservation status of the region. The study also raised the possibility that there may be an incipient species within *B. barratti*, restricted to eastern Zimbabwe, and further sampling of the fragmented populations within the *B. lopezi* complex are likely to reveal additional significant structure. In addition the possible hybridisation between *B. barratti* and *B. sylvaticus* in southern Kwa-Zulu Natal warrants further investigation.

Finally the most obvious extension of this research would be to analyse a larger number of geographically representative samples for both the *A. thoracica* complex and the African *Bradypterus* warblers. More extensive ingroup and outgroup sampling with inclusion of all 10 African *Bradypterus* species and some of the Asian species might prove to be more insightful in the relationships within *Bradypterus* and among the outgroups. This would fulfil the requirements for the initial proposed comparative phylogeographic study. In addition more intense sampling in the areas where the deep phylogeographic breaks are located in *A. thoracica* could shed some additional light on speciation in Afromontane forest birds as well as the biogeographic histories of these areas.

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Appendix I

Summary of the samples used in this phylogenetic study of the *A. thoracica* complex. Subspecies and corresponding sampling localities are indicated.

Subspecies	Locality	Co-ordinates	Type of Materrial	Collector	Date Collected
griseopyga 1	Lamberts Bay, SA	18°18'E 27°21'S	formalin	Tvl Mus	12/08/84
griseopyga 2	Near Yzerfontein, SA	18°07'E 33°20'S	frozen tissue	Durb Mus	30/08/91
griseopyga 3	Near Yzerfontein, SA	18°07'E 33°20'S	frozen tissue	Durb Mus	30/08/91
capensis 1	Swellendam, SA	20°27'E 34°04'S	frozen tissue	P. Ryan	07/03/99
capensis 2	Die Poort, Herbatsdale, SA	21°45'E 34°02'S	blood	P. Ryan	2028/03/01
clandei/capensis	Bosplaas near George, SA	22°28'E 33°56'S	frozen tissue	Durb Mus	05/09/91
clandei 1	Groenvlei nr Knysna, SA	22°52'E 34°02'S	blood	P. Ryan	2019/03/01
clandei 2	Natures Valley, SA	23°35'E 33°58'S	blood	P. Ryan	2028/03/01
thoracica	Gamtoos, SA	24°56'E 33°42'S	blood	P. Ryan	2027/03/01
venusta 1	Weza Forest, SA	29°45'E 30°35'S	blood	P. Ryan	2026/03/01
venusta 2	East London, SA	27°58'E 33°03'S	blood	P. Ryan	2027/03/01
darglensis 1	Boschoek near Howick, SA	30°14'E 29°28'S	frozen tissue	Durb Mus	18/02/92
drakensbergensis 1	Wakkerstroom, SA	30°07'E 27°13'S	Liver/DMSO	P. Ryan	2024/03/01
drakensbergensis 2	Waterval Boven, SA	30°30'E 25°35'S	heart/DMSO	P. Ryan	2023/03/01
lebomboensis	20km west of Pongola, SA	31°50'E 27°20'S	blood	P. Ryan	2024/03/01
spelonkensis 1	Levubu, SA	30°20'E 23°10'S	blood	C T Symes	19/11/99
spelonkensis 2	near Louis Trichardt, SA	29°44'E 23°03'S	blood	C. Killian	
flaviventris	Pretoria Botanical gardens, SA	28°10'E 25°42'S	blood	P. Ryan	2023/03/01
rhodesiae	Harrare, Zim	31°04'E 17°51'S	blood	C. Cohen	11/12/98
arnoldi	55km NW Mutare, Zim	32°40'E 18°56'S	blood	P. Ryan	2008/07/01

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Appendix I (continued.)

Subspecies	Locality	Co-ordinates	Type of Materrial	Collector	Date Collected
lynesi	Namuli, Moz	37°04'E 15°22'S	blood	P. Ryan	06/12/98
flavigularis	Zomba, Mal	35°20'E 15°45'S	blood	P. Ryan	
whitei	Mutinondo, Zam	31°30'E 02°00'S	blood	P. Ryan	
youngi	Nyika, Mal	34°00'E 10°25'S	blood	P. Ryan	2029/06/01
uluguru	Ulungu Mnts, Tanz	37°40'E 07°00'S	blood	Lab DM	06/10/93
murina	Poroto Mts, Tanz	33°30'E 09°02'S	blood	P. Ryan	2028/06/01
griseiceps	Mnt Meru, Tanz	37°40'E 00°15'N	blood	Lab DM	10/01/93
fuscigularis	Ngan, Taita, Kenya	38°20'E 03°25'S	blood	Lab DM	

Tvl Mus, Transvaal Museum; Durb Mus, Durban Museum of Natural History; SA, South Africa; Zim, Zimbabwe Moz, Mozambique; Mal, Malawi; Zam, Zambia; Tanz, Tanzania

Appendix II

Pairwise genetic distances between members of the *A. thoracica* complex and potentially related *Sylviidae*, based on 600 nucleotides of the cytochrome *b* gene. Above diagonal: HKY 85 distances (%) and below diagonal: HKY 85 + I (proportion invariable sites) + G (gamma shape distribution parameter) distances (%).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1	*A.t. gryseopyga 1*		0.2	2.9	3.1	3.0	2.9	3.0	4.5	4.3	4.3	5.1	5.2	4.9	6.2	5.8	5.9	4.9	6.1	5.4	5.1	6.9	6.0	7.3	6.2	6.9	6.4	10.6	13.3	16.2
2	*A.t. gryseopyga 2*	0.2		3.1	3.3	3.2	3.1	3.1	4.7	4.5	4.5	5.2	5.4	5.1	6.4	6.0	6.1	5.0	6.3	5.6	5.2	7.1	6.2	7.5	6.4	7.1	6.5	10.8	13.5	16.5
3	*A.t. capensis 1*	3.3	3.5		0.2	0.5	0.3	0.5	4.4	4.2	4.2	5.3	5.2	5.1	6.0	5.6	5.9	5.4	6.9	6.0	5.6	7.3	6.4	7.7	6.6	7.1	6.2	11.4	13.1	15.6
4	*A.t. capensis 2*	3.5	3.7	0.2		0.7	0.5	0.3	4.2	4.0	4.0	5.1	5.0	4.9	6.2	5.8	6.1	5.6	7.1	6.2	5.8	7.1	6.2	7.5	6.4	6.9	6.0	11.2	12.9	15.4
5	*capensis/clandei*	3.4	3.7	0.5	0.7		0.3	0.7	4.7	4.5	4.5	5.1	5.4	4.9	5.5	5.5	6.0	5.3	6.8	5.8	5.5	7.4	6.4	7.7	6.6	7.2	6.6	11.3	13.5	15.5
6	*A.t. clandei*	3.3	3.5	0.3	0.5	0.4		0.5	4.7	4.5	4.5	4.9	5.0	4.7	5.6	5.2	5.7	5.1	6.5	5.6	5.2	6.9	6.0	7.3	6.2	6.7	6.2	11.0	12.9	15.2
7	*A.t.thoracica*	3.3	3.6	0.5	0.3	0.7	0.5		4.2	4.1	4.1	4.8	5.0	4.6	5.9	5.5	6.1	5.3	6.8	5.9	5.5	7.1	6.1	7.5	6.3	6.5	6.3	11.0	13.2	15.0
8	*A.t.venusta 1*	5.4	5.7	5.2	5.0	5.7	5.8	5.1		0.2	0.2	5.8	5.9	6.0	7.3	6.9	6.5	6.5	8.1	6.9	6.5	8.3	6.9	7.9	7.1	7.3	6.7	10.8	14.6	16.7
9	*A.t.venusta 2*	5.2	5.4	5.0	4.7	5.5	5.5	4.8	0.2		0.0	5.6	5.7	5.8	7.1	6.7	6.3	6.4	7.9	6.7	6.4	8.1	6.7	8.1	6.9	7.5	6.5	10.6	14.4	16.5
10	*A.t. darglensis 1*	5.2	5.4	5.0	4.7	5.5	5.5	4.8	0.2	0.0		5.6	5.7	5.8	7.1	6.7	6.3	6.4	7.9	6.7	6.4	8.1	6.7	8.1	6.9	7.5	6.5	10.6	14.4	16.5
11	*A.t. drakensbergensis 1*	6.2	6.5	6.5	6.2	6.2	6.0	5.8	7.3	7.0	7.0		0.2	0.2	2.0	2.0	1.3	2.7	4.2	2.7	2.4	4.9	3.4	4.5	3.6	4.2	4.0	10.2	13.4	15.3
12	*A.t. drakensbergensis 2*	6.4	6.7	6.4	6.1	6.8	6.1	6.1	7.5	7.1	7.1	0.2		0.0	1.6	1.8	1.4	2.7	3.9	2.9	2.5	4.4	3.1	4.6	3.3	4.4	4.2	9.6	12.9	14.7
13	*lebomboensis*	5.9	6.2	6.2	6.0	6.0	5.7	5.5	7.6	7.3	7.3	0.2	0.0		2.2	2.2	1.5	2.6	4.0	2.6	2.2	4.7	3.3	4.3	3.4	4.0	3.8	10.2	13.2	15.1
14	*A.t. spelonkensis 1*	7.9	8.2	7.6	8.0	6.8	7.0	7.5	9.9	9.5	9.5	2.2	1.7	2.4		0.7	0.6	3.8	5.3	3.8	3.1	5.1	4.2	5.0	4.3	4.9	4.7	11.2	14.2	16.7
15	*A.t. spelonkensis 2*	7.3	7.6	7.1	7.4	6.8	6.5	6.9	9.2	8.9	8.9	2.2	2.0	2.4	0.7		0.8	3.4	4.5	3.8	3.1	4.7	4.2	5.4	4.3	4.5	4.3	11.0	13.8	16.2
16	*A.t. flaviventris*	7.3	7.7	7.4	7.8	7.6	7.1	7.8	8.4	8.0	8.0	1.4	1.5	1.6	0.6	0.8		3.5	4.9	3.7	2.9	5.1	4.3	5.1	4.5	5.1	4.9	11.0	13.5	16.5
17	*A.t. rhodesiae*	5.8	6.1	6.7	7.0	6.5	6.1	6.6	8.5	8.1	8.1	3.0	2.9	2.8	4.4	3.9	3.9		2.0	1.3	2.2	5.1	3.4	5.4	3.6	4.2	3.6	10.3	13.3	15.1
18	*A.t. arnoldi*	7.6	8.0	9.0	9.4	8.8	8.4	8.9	11.1	10.7	10.7	4.8	4.5	4.6	6.3	5.3	5.7	2.2		2.0	3.3	3.8	4.0	5.8	4.1	5.1	4.9	10.6	13.4	14.8
19	*A.t. whitei*	6.6	6.9	7.6	7.9	7.3	7.0	7.5	9.1	8.8	8.8	3.0	3.2	2.8	4.4	4.4	4.1	1.4	2.2		1.9	4.0	2.9	4.5	2.7	3.6	3.4	10.6	13.5	14.5
20	*A.t. lynesi*	6.1	6.4	7.1	7.4	6.8	6.5	6.9	8.5	8.2	8.2	2.6	2.8	2.4	3.5	3.5	3.2	2.4	3.6	2.0		4.2	2.4	4.1	2.6	3.5	2.9	10.0	13.1	14.7

Appendix II (continued)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
21 *A.t. flavigularis*	9.0	9.4	9.9	9.5	10.0	9.2	9.4	11.6	11.2	11.2	5.9	5.2	5.7	6.1	5.6	6.1	6.2	4.4	4.7	4.9		1.7	4.3	1.5	3.8	3.6	9.4	13.2	14.6
22 *A.t. youngi*	7.6	7.9	8.3	8.0	8.4	7.7	7.9	9.2	8.8	8.8	3.9	3.5	3.7	4.9	4.9	5.0	4.0	4.6	3.3	2.6	1.8		3.0	0.2	2.4	2.2	9.0	12.7	14.1
23 *A.t. uluguru*	9.9	10.2	10.8	10.4	10.8	10.0	10.3	11.0	11.4	11.4	5.3	5.6	5.1	6.1	6.7	6.2	6.7	7.1	5.2	4.8	5.0	3.4		2.8	2.6	3.6	9.7	11.3	13.7
24 *A.t. murina*	7.9	8.2	8.6	8.3	8.8	8.0	8.2	9.5	9.2	9.2	4.2	3.8	3.9	5.1	5.2	5.3	4.2	4.8	3.0	2.8	1.6	0.2	3.1		2.2	2.0	9.0	12.5	13.9
25 *A.t. griceiceps*	9.2	9.6	9.7	9.3	9.9	9.0	8.5	9.9	10.3	10.3	4.9	5.3	4.7	5.9	5.4	6.2	5.0	6.1	4.2	4.0	4.5	2.7	2.9	2.4		2.2	8.4	11.9	14.1
26 *A.t. fuscigularis*	8.2	8.6	8.0	7.7	8.8	8.0	8.2	8.8	8.5	8.5	4.7	5.0	4.4	5.7	5.2	5.9	4.2	5.9	3.9	3.3	4.2	2.4	4.1	2.2	2.4		8.6	11.9	14.3
27 *A.flavigularis*	16.7	17.3	19.5	18.9	19.1	18.3	18.2	17.5	17.0	17.0	15.6	14.3	15.6	18.4	17.7	17.3	15.9	16.5	16.6	15.0	14.0	12.9	14.4	12.8	11.6	12.0		10.8	15.3
28 *Cisticola*	23.7	24.6	23.4	22.7	24.4	22.7	23.6	28.7	27.8	27.8	24.2	23.2	23.4	27.2	25.6	24.7	23.8	24.1	24.2	23.1	23.5	22.0	18.0	21.2	19.3	19.4	15.8		13.7
29 *Prinia*	34.7	36.2	31.9	30.8	31.3	29.8	29.0	38.1	36.7	36.7	30.0	26.9	29.1	36.4	34.2	34.7	29.2	28.5	26.8	27.6	27.8	25.4	23.7	24.6	25.4	26.4	29.1	23.8	

Appendix III

Estimate times of divergence for mitochondrial DNA haplotypes for *Apalis thoracica*, based on a molecular clock of 2% sequence divergence per million years.

Comparison	Time of divergence (Ma)	Epoch	Comparison	Time of divergence (Ma)	Epoch
Within sSA :			*murina* vs. :		
griseopyga vs. *thoracica*	1.5 -1.6	Pleistocene	*fuscigularis*	1.0 - 1.1	Pleistocene
griseopyga vs. *venusta*	2.2 - 2.1	Pliocene	*uluguru*	1.4 - 1.5	Pleistocene
thoracica vs. *venusta*	2.0 - 2.4	Pliocene	*griseiceps*	1.1 - 1.2	Pleistocene
			flavigularis	0.8	Pleistocene
Within nSA :			*arnoldi*	2.0 - 2.1	Pliocene
drakensbergensis vs. *spelonkensis*					
			fuscigularis vs. :		
nSA vs. :			*griseiceps*	1.1	Pleistocene
lynesi	1.1 - 1.5	Pleistocene	*uluguru*	1.8	Plio-Pleistocene
whitei	1.3 - 1.9	Plio-Pleistocene	*flavigularis*	1.8	Plio-Pleistocene
murina	1.6 - 2.2	Plio-Pleistocene	*arnoldi*	2.4	Pliocene
fuscigularis	1.9 - 2.4	Pliocene			
uluguru	2.1 - 2.7	Pliocene	*uluguru* vs.:		
griseiceps	2.0 - 2.3	Pliocene	*griseiceps*	1.3	Pleistocene
arnoldi	2.0 - 2.6	Pliocene	*arnoldi*	2.9	Pliocene
flavigularis	2.2 - 2.5	Pliocene	*flavigularis*	2.2	Pliocene
whitei vs. :			*griseiceps* vs.:		
lynesi	0.9 - 1.1	Pleistocene	*arnoldi*	2.5	Pliocene
murina	1.4 - 1.8	Pleistocene	*flavigularis*	1.9	Pliocene
fuscigularis	1.7 - 1.8	Pleistocene			
arnoldi	1.0	Pleistocene	*arnoldi* vs. *flavigularis*	1.9	Plio-Pleistocene
griseiceps	1.8 - 2.1	Plio-Pleistocene			
uluguru	2.2 - 2.7	Pliocene			
flavigularis	2.0 - 2.5	Pliocene			
lynesi vs. :					
murina	1.2 - 1.3	Pleistocene			
fuscigularis	1.5	Pleistocene			
griseiceps	1.7	Pleistocene			
arnoldi	1.6	Pleistocene			
uluguru	2.0	Pliocene			
flavigularis	2.1	Pliocene			

Appendix IV

Aligned nucleotide sequences of the mtDNA cytochrome *b* gene for the *Apalis thoracica* complex. Included are corresponding sequences for *Prinia bairdi*, *Cisticola juncidis* and *Apalis flavida* that were used as outgroups in phylogenetic analysis, see Fig. 3). The sequences correspond to positions 15085-15684 of the chicken mitochondrial genome (Desjardins & Morais 1990). Dots indicate identity with first line of each block. Unknown nucleotides are marked by '?'.

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                     111111111122222222223333333333444444444455555555556666666666777777777788888888889999999999000000000011111111112222222222333333
Taxon/Node  123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345
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griseop 1   TCCTCCGTAACCCACATGTGCCGAGATGTCCAATTCGGCTGACTAATTCGCAATCTACATGCAAACGGAGCCTCATTCTTCTTTATCTGCATCTACCTACACATCGGCCGAGGCTTCTACTACGGCTCATACCTA
griseop 2   .................................................A.....................................................................................
capens  1   ..........................C........................................................C................................T..................
capens  2   ..........................C........................................................C................................T..................
cap/clan    ???????...................C...........T............................................C................................T..................
clandei     ..........................C........................................................C................................T..................
thoraci 1   ..........................C........................................................C................................T..................
venusta 1   ..........................C........................................................C.............................T..T..................
venusta 2   ..........................C........................................................C.............................T..T..................
darglen 1   ..........................C........................................................C.............................T..T..................
drakens 1   ...........T..............C........................................................C................................T..................
drakens 2   ?????????????????????????.C........................................................C................................T..................
lebombo     ...........T..............C........................................................C................................T..................
spelonk 1   ??T..G....................C..A.....................................................C................................T..................
spelonk 2   ..........................C..A.....................................................C................................T..................
flaviven    .....G.....T..............C..A.....................................................C................................T..................
rhodesia    ..........................C.....G..................................................C................................T..................
arnoldi     ........C.................C.....G....................C.............................C........T.......................T..................
whitei      ..........................C.....G..................................................C................................T..................
lynesi      ..........................C..........................C.............................C................................T..................
flavigul    ..........................C..........................C.............................C................................T..................
youngi      ..........................C..........................C.............................C................................T..................
uluguru     ??????????????????........C..........................C....................C........C................................T..................
murina      ..........................C..........................C.............................C................................T..................
griseic     ..........................C....................C.....C.............................C................................T..................
fuscigul    ..........................C..........................C.............................C................................T............C.....
A.flavida   G........G.T.....A.....T..C........T...........C.....C...........T.................C......................................T............
Cisticola   ........CG.T.....A........C...........A........C.....C.....C.....T........C..........................T...........A.....................
Prinia      ........C.....TG.A........C.....G..T........G..C.....C..G.................G........C.....T........T..T...........A........T...........C
```

Appendix IV (continued)

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             111111111111111111111111111111111111111111111111111111111111111122222222222222222222222222222222222222222222222222222222222222222222222
             333344444444445555555555666666666677777777778888888888999999999900000000001111111111222222222233333333334444444444555555555566666666667
Taxon/Node   678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
------------------------------------------------------------------------------------------------------------------------------------------------------
griseop 1    AACAAAGAAACCTGAAACGTAGGAGTCATCCTATTACTAACCCTCATAGCAACTGCCTTCGTAGGATATGTACTTCCCTGAGGACAAATGTCATTCTGAGGAGCAACAGTCATTACAAACCTATTCTCAGCCATC
griseop 2    .......................................................................................................................................
capens  1    ..............................T..C....G..............C..............C.....A..............A.............................................
capens  2    ..............................T..C....G..............C..............C.....A..............A.............................................
cap/clan     ..............................T..C.....G.............C..............C.....A..............A.............................................
clandei      ..............................T..C...................C..............C.....A..............A.............................................
thoraci 1    ..............................T..C...................C..............C.....A..............A.............................................
venusta 1    ..............................T.......G.....A.................G..G..C.....A........G...................................................
venusta 2    ..............................T.......G.....A.................G..G..C.....A........G...................................................
darglen 1    ..............................T.......G.....A.................G..G..C.....A........G...................................................
drakens 1    ................................CC.......T....................G..G..C.....A............................................................
drakens 2    ................................CC.......T....................G.....C.....A............................................................
lebombo      ................................CC.......T....................G.....C.....A............................................................
spelonk 1    ................................CC.......T...........C........G..G..C.....A..............A.......................C.....................
spelonk 2    ................................CC.......T...........C........G..G..C.....A..............A.......................C.....................
flaviven     ................................CC.......T...........C........G..G..C.....A......................................C.....................
rhodesia     ................................CC............................T.....C.....A............................................................
arnoldi      ................................CC............................T.....C.....G............................................................
whitei       ................................CC............................G.....C.....A............................................................
lynesi       ................................CC............................G.....C.....A.......................G..............C.....................
flavigul     ................................CC..................................C.....G..............A.......................C..G..................
youngi       ................................CC............................G.....C.....G..............A.......................C..G..................
uluguru      .................T........T.....CC.......T....................G.....C.....G..............A.......................C..G..................
murina       ................................CC............................G.....C.....G..............A.......................C..G..................
griseic      ................................CC.......T....................G.....C.....A..............A...........G...........C..G..................
fuscigul     ................................CC....G.................T.....G.....C.....A..............A.......................C..G..................
A.flavida    ........G...................................G...........T........C..C.....G..............A..G........G...........C.....................
Cisticola    ...........G..............?.....CC....T.....T........C..T...........C..C..G..............A...........G...........C....................T
Prinia       ..T.......................A.....CC..........G.....G..A.....T..G.....C..C..G..........................G...........C.................A...
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Appendix IV (continued)

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            222222222222222222222222222223333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333444444
            777777777888888888899999999990000000000111111111122222222223333333333444444444455555555556666666666777777777788888888889999999999000000
Taxon/Node  123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345
-----------------------------------------------------------------------------------------------------------------------------------------------
griseop 1   CCCTACATCGGACAGACACTAGTAGAATGGGCATGAGGCGGATTCTCTGTAGATAACCCAACACTCACCCGATTCTTCGCCATTCATTTCCTACTCCCATTCATCATTGCAGGGCTGACACTCGTACACCTCACC
griseop 2   .......................................................................................................................................
capens  1   .............................A......................................T.................C..........................A.....................
capens  2   .............................A......................................T.................C..........................A.....................
cap/clan    .............................A......................................T.................C..........................A.....................
clandei     .............................A......................................T.................C..........................A.....................
thoraci 1   .............................A......................................T.................C.................T........A.....................
venusta 1   ........T..G...................................C.....C..............T..............C..C.........................................T......
venusta 2   ........T......................................C.....C..............T..............C..C.........................................T......
darglen 1   ........T......................................C.....C..............T..............C..C.........................................T......
drakens 1   ..................T..................................C.............................C........C..............C..G..A..A..................
drakens 2   ..................T..................................C.............................C........C..............C..G..A..A..................
lebombo     ..................T..................................C.............................C........C..............C..G..A..A..................
spelonk 1   ..................T..................................C.............................C........C..............C..G..A..A.....T............
spelonk 2   ..................T..................................C.............................C........C..............C.....A..A.....T............
flaviven    ..................T..................................C.............................C........C..............C..G..A..A.....T............
rhodesia    .......................T.............................C....................T.....T..C........C..............C.....A..A..................
arnoldi     ..A....................T..G..........................C....................T.....T..C........C..............C.....A..A........G.........
whitei      ..A....................T..G..........................C...........................T..C........C.........G....C..G..A..A........G.........
lynesi      .......................C.............................C.............................C........C..............C..G..A..A........G.........
flavigul    ..A....................T..G..............G.....C.....C.................G...........C........C.........G....C..G..A..A.....T..C.........
youngi      .......................T..G....................C.....C.............................C........C..............C..G..A..A........C.........
uluguru     ...........G..A...........G...........T........C.....C.............................C........C.........G....C..G..A..A........T.........
murina      .......................T..G....................C.....C.............................C........C.........G....C..G..A..A........C.........
griseic     ...........G..A........T..G....................C.....C........................T.....C........C.........G.T..C.....A..A........C.........
fuscigul    .......................T.......................C.....C.............................C........C.........G....C.....A..A........C.........
A.flavida   ..A.....T.....A................................C.....C..T..C.................T.....C..C.....C.........C....C.....A.....G.....C.........
Cisticola   ........T.....A...T..........A........T........A........T.....C..............T..T..C..C.....C..T.....TG..........A..A........C...T.A...
Prinia      ..A...G.......A...T....C.....A..C..............A.....C..............T...........T.....C........G......G....C.T...A..A.....A..C.........
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Appendix IV (continued)

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               4444444444444444444444444444444444444444444444444444444444444444444444444444444444444444444444555555555555555555555555555555555555555555
               0000111111111122222222223333333333444444444455555555556666666666777777777788888888889999999999000000000011111111112222222222333333333344
Taxon/Node     6789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901
-------------------------------------------------------------------------------------------------------------------------------------------------------
griseop 1      CTCCTTCATGAATCCGGATCAAACAACCCGCTAGGAATCCCATCAGACTGTGACAAAATCCCATTCCACCCCTACTACTCCACAAAGGACATCCTTGGATTCGCACTACTATTCATCCCCCTTGCTGGCCTAGCCT
griseop 2      ........................................................................................................................................
capens  1      .....................................................................................................T..............T...................
capens  2      .............................A.......................................................................T..............T...................
cap/clan       .....................................................................................................T..............T...................
clandei        .....................................................................................................T..............T...................
thoraci 1      .............................A.......................................................................T..............T...................
venusta 1      ........C....................AT.........................................................................................................
venusta 2      ........C....................AT.........................................................................................................
darglen 1      ........C....................AT.........................................................................................................
drakens 1      ........C........G...........A.........................................T........T..T..............G.................T...................
drakens 2      ........C........G...........A.........................................T........T..T..............G.................T...????????????????
lebombo        ........C........G...........A.........................................T........T..T..............G.................T...................
spelonk 1      ........C........G.....................................................T........T..T..............G.....................................
spelonk 2      ........C........G.....................................................T........T..T..............G.....................................
flaviven       ........C........G.....................................................T........T..T..............G...............................??????
rhodesia       .....C..C...........................................................T..T........T..T..............G.....................................
arnoldi        ........C...........................................................T..T........T..T...........?..G.........A.........................T.
whitei         .....C..C...........................................................T..T........T..T..............G.....................................
lynesi         .....C..C..........................G...................................T........T..T..............G.....................................
flavigul       ........C....................A.........................................T........?..T......G.......G.........A................C...T....T.
youngi         .....C..C....................A.........................................T........T..T......G.......G..........................C...T......
uluguru        .....C..C.....T..............A....................C....................T...........T..............G.....................................
murina         .....C..C....................A.........................................T........T..T......G.......G..........................C...T......
griseic        .....C..C....................A....................C....................T........T..T..............G.....................................
fuscigul       .....C..C....................A....................C....................T........T.TT..A...........G.....................................
A.flavida      .....A..C..G.................A....................C.............................T..G..A........C..G.........A..........T..A..CA........C
Cisticola      .....C..C...A.A..............AA........................................T..............A.....T..A............A..C......TT..A...A.......AC
Prinia         ..A..C..C...A.T.....C........A..T.....T...........C........T...........T..T...........A......G.A.........T..A..C.A..T.T......CA..T.....C
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Appendix IV (continued)

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             55555555555555555555555555555555555555555555555555555555556
             44444444555555555566666666667777777777888888888899999999990
Taxon/Node   23456789012345678901234567890123456789012345678901234567890
-----------------------------------------------------------------------
griseop 1    TATTCGCCCCTAACCTGCTAGGAGACCCAGAAAACTTCACACCAGCTAACCCACTAGCC
griseop 2    ...........................................................
capens  1    .....................................................T.....
capens  2    .....................................................T.....
cap/clan     .............................................??????????????
clandei      ..............................................C......T.....
thoraci 1    .................................................??????????
venusta 1    ...................G.....T................................T
venusta 2    ...................G.....T................................T
darglen 1    ...................G.....T................................T
drakens 1    ..............................................C..T.........
drakens 2    ???????????????????????????????????????????????????????????
lebombo      ..............................................C..T.........
spelonk 1    ..........C.............................G.....C..T.........
spelonk 2    ..........C......T......................G.....C..T.........
flaviven     ???????????????????????????????????????????????????????????
rhodesia     ................A.............................C..T.........
arnoldi      .................T............................C..T.........
whitei       ..............................................C..T.........
lynesi       ..............................................C..T.........
flavigul     ..........?......T............................C............
youngi       ..............................................C............
uluguru      .....T........................................C............
murina       ..............................................C............
griseic      ..............................................C............
fuscigul     ..............................................C............
A.flavida    ................A....................T........C...........T
Cisticola    .....T.T........C.....T..............T........C............
Prinia       .....T.A..............C.......................C........G..T
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Appendix V

Species and geographical distributions of the African Bradypterus warblers included in this study.

Species	Locality	Co-ordinates	Type of materrial	Collector	Date collected
B. b. baoecala					
B. b. transvaalensis (1)	Pilanesberg National Park, South Africa	27°10'E 25°15'S	blood	L. Solms	
B. b. transvaalensis (2)	50 kilometers NW of Mutare, Zimbabwe	33°10'E 19°12'S	blood	P. Ryan	07/80/2001
B. b. tongensis (1)	Rumpi-Nyika, Malawi	34°00'E 10°25'S	blood	P. Ryan	
B. b. tongensis(2)	Kasanka, Zambia	30°05'E 13°00'S	blood	P. Ryan	
B. carpalis	Lake Kanydoli, W Kenya	34°09'E 04°00'N	blood	R. Bowi	12/06/1999
B. lopezi	Namuli, Mozambique	37°04'E 15°22'S	blood	P. Ryan	02/12/1998
	Zomba, S Malawi	35°20'E 15°45'S	blood	P. Ryan	07/12/1998
B. barratti godfreyi (1)	Howick, South Africa	30°14'E 29°28'S	tissue stored at -20	A. Berruti	18/02/1992
B. barratti godfreyi (2)	Weza, Ngele Forest, South Africa	29°45'E 30°35'S	tissue/DMSO	C. Symes	19/12/1998
B. barratti priesti (3)	Vumba, Zimbabwe	32°47'E 19°04'S	blood	P. Ryan	07/09/2001
B. sylvaticus sylvaticus (1)	George, South Africa	22°28'E 33°56'S	tissue stored at -20	A. Berruti	05/09/1991
B. sylvaticus pondoensis (2)	East London, South Africa	27°58'E 33°03'S			

Appendix V (continued)

Species	Locality	Co-ordinates	Type of materrial	Collector	Date collected
B. victorini	Outeniqua Mts, South Africa (2 individuals)	23°00'E 33°50'S	tissue stored at -20	A. Berruti	06/09/1991
	Diepvalle, Knysna, Souht Africa	23°03'E 34°04'S	tissue/DMSO	P. Ryan	20/03/2001

*Numbers in parentheses refer to taxon numbers in Table 5
†Co-ordinates are approximate measures

Appendix VI

Aligned nucleotide sequences of the mtDNA cytochrome *b* gene of twelve *Bradypterus* taxa plus *Sylvia melanocephala*, *Cettia cetti*, *Acrocephalus aedon* and *Phylloscopus laetus* (used as outgroups in phylogenetic analysis, see Fig. 3). The sequences correspond to positions 15073-15676 of the chicken mitochondrial genome (Desjardins & Morais 1990). Dots indicate identity with first line of each block. Unknown nucleotides are marked by '?'.

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                                                                                                                       11111111111111111111111111111
                             1111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990000000000111111111122222222
Taxon/Node          12345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678
---------------------------------------------------------------------------------------------------------------------------------------------------
tongensis 1         GCCCTAGCCTTCTCATCCGTAGCTCATCTATGCCGAAACGTCCAATTCGGTTGACTTATCCGAAACTTACATGCAAACGGAGCCTCCCTATTCTTCATCTGCATCTACTTCCACATCGGCCGAGGGAT
tongensis 2         ................................................................................................................................
transvaalensis 1    ................................................................................................................................
transvaalensis 2    ................................................................................................................................
carpalis            ?????????????????????????????????????????????????????????????.....C....C.........A...T...C.............................G.....A..
sylvaticus 1        ........T...........C..C..CA.............A.....T..C..............TC....C.................C...........T..T....................A..
sylvaticus 2        ........T...........C..C..CA...................T..C..............TC....C.................C...........T..T....................A..
lopezi              ....................T..C..CA.C.................T..............G...C....C.................C......G............................A..
barratti 1          ........T...........C..C..CA.............A.....T..C..............TC....C.................C...........T..T....................A..
barratti 2          ....................T..C..CA.G......G.............C.....C.....G...C....C.................C..........................T........A..
barratti 3          ....................T..C..CA.G....................C.....C.....G...C....C.................C..........................T........A..
victorini           T.......T...G.T.....CT.C..CA.C......G....A........C...T.A.........C....C..............AA................T...A.......T..A.....AT.
Sylvia              T.AT.G........C..T.....C..CA........G..........T..C.....A.........C.T..C...........T..AT.C..................A.T.....T........A..
Acrocephalus        T.T.....A...A.C.....C..C...G....TG..G....A..G..T..A...T.A.....C...C.T..................T.C.................T........T........AT.
Cisticola           T.T...........C.....C.....CA........G.............A.....A.....C...C....C.....T.........T.C.....T............C.A..T...........T..
Phylloscopus        T.......A...A.T..T..C..C..CAC............T........C.....A..T......C.C..C...............T.T.....T.................T...........AT.
Cettia              T.T........TG.C.....CA.C..CA.G........T........T........A........TC................T...T.C..T................................CC.
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Appendix VI (continued)

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                    11111111111111111111111111111111111111111111111111111111111111111111111222222222222222222222222222222222222222222222222222222222
                    23333333333444444444455555555556666666666777777777788888888889999999999000000000011111111112222222222333333333344444444445555555
Taxon/Node          90123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456
----------------------------------------------------------------------------------------------------------------------------------------------------
tongensis 1         CTACTACGGCTCATACCTAAACAAAGAAACCTGAAATGTCGGAGTTATCCTTCTACTCGCACTAATAGCAACTGCTTTCGTAGGCTACGTATTACCCTGAGGACAAATATCATTCTGAGGGGCTACCG
tongensis 2         ................................................................................................................................
transvaalensis 1    ................................................................................................................................
transvaalensis 2    ................................................................................................................................
carpalis            .........T.GG..........................T......G..........A.................................C......................T.....A.....A.
sylvaticus 1        ....................................CA.T...........C.....AA..........T..C...........T......C....................................
sylvaticus 2        ....................................CA.T...........C.....AA..........T..C...........T......C....................................
lopezi              ...T........T.....G........G........C.................C..AAT............C..................C.G........T.........................
barratti 1          ....................................CA.T...........C.....AA..........T..C...........T......C....................................
barratti 2          ...T.................T..............C........C........T..GTT...............................C....T.....C.........................
barratti 3          ...T.................T.......................C........T..GTT...............................C....T.....C.........................
victorini           .........T....................A.....CA.T.....A.....C..T..ACTT..C...........C...........T...C.T..........................A.....A.
Sylvia              ...........................G...........A...........G.....AA.C..C..G..C..............T.....TC.C..A........G..............A.....A.
Acrocephalus        .........A..C.......................CA....C...........G..A..C..C..G..C.....C........A..T..CC.G..A.......................A.....A.
Cisticola           ..............................G.....C..A.....G.....A.....TA.C...........C...........T.....CC.C................................A.
Phylloscopus        .........A..G..T....................CA.............A...T.AA....C.....C.....C.....C..G......C..........G....................C..A.
Cettia              A..T........T...T....................A.......A..T..C...T.A.................C..T.....T..T...C.C..A..............C..............A.
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Appendix VI (continued)

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                          22222222222222222222222222222222222222222223333333333333333333333333333333333333333333333333333333333333333333333333333333333333333
                          55566666666667777777777888888888899999999990000000000111111111122222222223333333333444444444455555555556666666666777777777788888
Taxon/Node                78901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234
-----------------------------------------------------------------------------------------------------------------------------------------------------
tongensis 1               TGATCACAAACCTATTCTCAGCAATCCCCTACATTGGCCAAACCCTAGTAGAATGAATATGAGGAGGATTCTCAGTAGACAACCCCACATTAACTCGATTCTTCGCCATCCACTTCCTTCTCCCATTC
tongensis 2               ................................................................G...............................................................
transvaalensis 1          ................................................................G...............................................................
transvaalensis 2          ................................................................G...............................................................
carpalis                  .A.....G....................A..T..C.......................................................C....C........T........T.....C..T......
sylvaticus 1              .A................................C.........T...................C....................T..GC.C..C..C.....T..TC..........C.........
sylvaticus 2              .A................................C.........T...................C........G...........T..GC.C..C..C.....T..TC..........C.........
lopezi                    .A..T.......................A...........G.........................................T..A...C....C.......................C.........
barratti 1                .A................................C.........T...................C....................T..GC.C..C..C.....T..TC..........C.........
barratti 2                ..................................C.........T...................T.................T......C...............................A......
barratti 3                ......................T...........C.........T.......G...........T.................T......C...............................A......
victorini                 .C.....C........A...........A..............A............GC......T....................A..CC...................T........C..A.....T
Sylvia                    .A..T..............T..T.....A.....C..T.....A............GCC.......C.....G...............C....C...........TC.T........C.....C...
Acrocephalus              .A..T........C.....T..T.....A..............A..G.........GC.........C..T..............A..CC....C........T..............C.........
Cisticola                 .C..?......T..........C..............A.....AT...........GC......T..............T..T..A..CC.C..C....C...T..T...........C..T.....T
Phylloscopus              .A..T.......................A..............A............GC......C.......................CC.................C....................
Cettia                    .A.....C....................A........T..............G...C.......C...........T..T.....A..CC....C.....T........T..T.....A..T......
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Appendix VI (continued)

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                     33333333333333334444444444444444444444444444444444444444444444444444444444444444444444444444444444444444444444444445555555555555
                     88888999999999990000000000111111111122222222223333333333444444444455555555556666666666777777777788888888889999999999000000000011l
Taxon/Node           56789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012
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tongensis 1          GTCATTGCAGGACTAACACTAGTCCACCTCACCCTTCTACACGAAACAGGCTCCAACAACCCTCTAGGCATCCCCTCGGACTGTGACAAAATCCCATTCCACCCATACTACTCCACAAAAGATATCCT
tongensis 2          ................................................................................................................................
transvaalensis 1     ................................................................................................................................
transvaalensis 2     ................................................................................................................................
carpalis             .....C.....G.....G.........................................T........A........A..T..............................A..........C.....
sylvaticus 1         .................C..............A.....C...........A...........C..............A..T..C...........................A.T.....G..C.....
sylvaticus 2         .................C..............A.....C...........A...........C..............A..T..C...........................A.T.....G..C.....
lopezi               .......................T..............G....................T..C...........T..A.....C..T.....T.............................C..T..
barratti 1           .................C..............A.....C...........A...........C..............A..T..C...........................A.T.....G..C.....
barratti 2           .....C.....G..G............................................T..C...........T..A..T..C..T....................T...A..........C.....
barratti 3           .....C.....G..G.........................................T.....C...........T..A..T..C..T....................T...A..........C.....
victorini            ............G.C...........T..A...T.C.....T........A..A........AAC...AG.G.....A.....C.......................T...A.......G..C.....
Sylvia               ...........C......T....A..T........A.........T....G..T........C..............A..T..C....................C.......................
Acrocephalus         A....C.....GT.C..T...........A.....C.....T..T.....A..A.........T....A..T..A..A..T..C....................T........AGT......C..TT.
Cisticola            ....................C......T.A?....C..C...........A..A........AA....A.....A..A........................A.T.................C..T..
Phylloscopus         A..........G..C..C.....G.....A.....A..............A..A........C.....A..T..A..A.....C....................C........T........C..T..
Cettia               .....C.T......C.................AT.C...........G..T..T........CA....A..T..A..A.....C.................T.....T.....T.TT.....C...T.
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Appendix VI (continued)

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                   5555555555555555555555555555555555555555555555555555555555555555555555555555555555555556666
                   1111111222222222233333333334444444444555555555566666666667777777777888888888899999999990000
Taxon/Node         3456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123
-----------------------------------------------------------------------------------------------------------------
tongensis 1        AGGGTTCGCACTCACACTTATCCTACTGGTCAGCTTAGCACTCTTCCTACCCAACTTCCTGGGCGACCCGGAAAATTTCACCCCAGCCAAC
tongensis 2        ...........................................................................................
transvaalensis 1   ..............................................................................T............
transvaalensis 2   ...A..........................................................................T............
carpalis           ...A..............C.....G..A......C..................................A.....C..........?????
sylvaticus 1       ...A..T.....T.....C.C...C..A....................G......C....A..T.....A.....C...............
sylvaticus 2       ...A..T.....T.....C.C...C..A....................G......C....A..T.....A.....C...............
lopezi             ...A........A.....C........A..AG..C....................C.T..A..T..T..A.....C...........T...
barratti 1         ...A..T.....T.....C.C......A....................G..T...C....A..T.....A.....C..........?????
barratti 2         ...A..............C.C......A..AG..C....G..T........T...C.T..A........A.....C...........T...
barratti 3         ...A..............C..T.....A..AG..C....G..T........T...C.G..A........A.....C...........T..T
victorini          ...A........A.T.A...C......T...TC.C....C..A...TCC..T...A.G..A..A.....A.....C.....A.........
Sylvia             ...C..T.....T.T.T.C....CT..C.C.TC.C.......A...GCC..T...C....A..A.....A.....C.....G..C......
Acrocephalus       ...A..T.T.....T...C.C...C..C.CTGCTC....C..A...GCC..T...C.AT.A..A.....A.....C.....A.........
Cisticola          ...A........A.T...C.....T..A.CT...C.......A...TCT..T...C....A..T.....A.....C..T..A.........
Phylloscopus       ...C..........T...A.....C..T.C.TC.C....C..A...TC...T...C.A..A..A.....A.....C.....G..G......
Cettia             ...A..T...T...T....G..TCT..C..TGC.C....C..A...TCC......C.GT.A..A.....A.....C.....A........T
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