Taxonomy, phylogeny and eco-biogeography of southern African white-eyes (*Zosterops* spp.) Aves: Order Passeriformes Family: Zosteropidae

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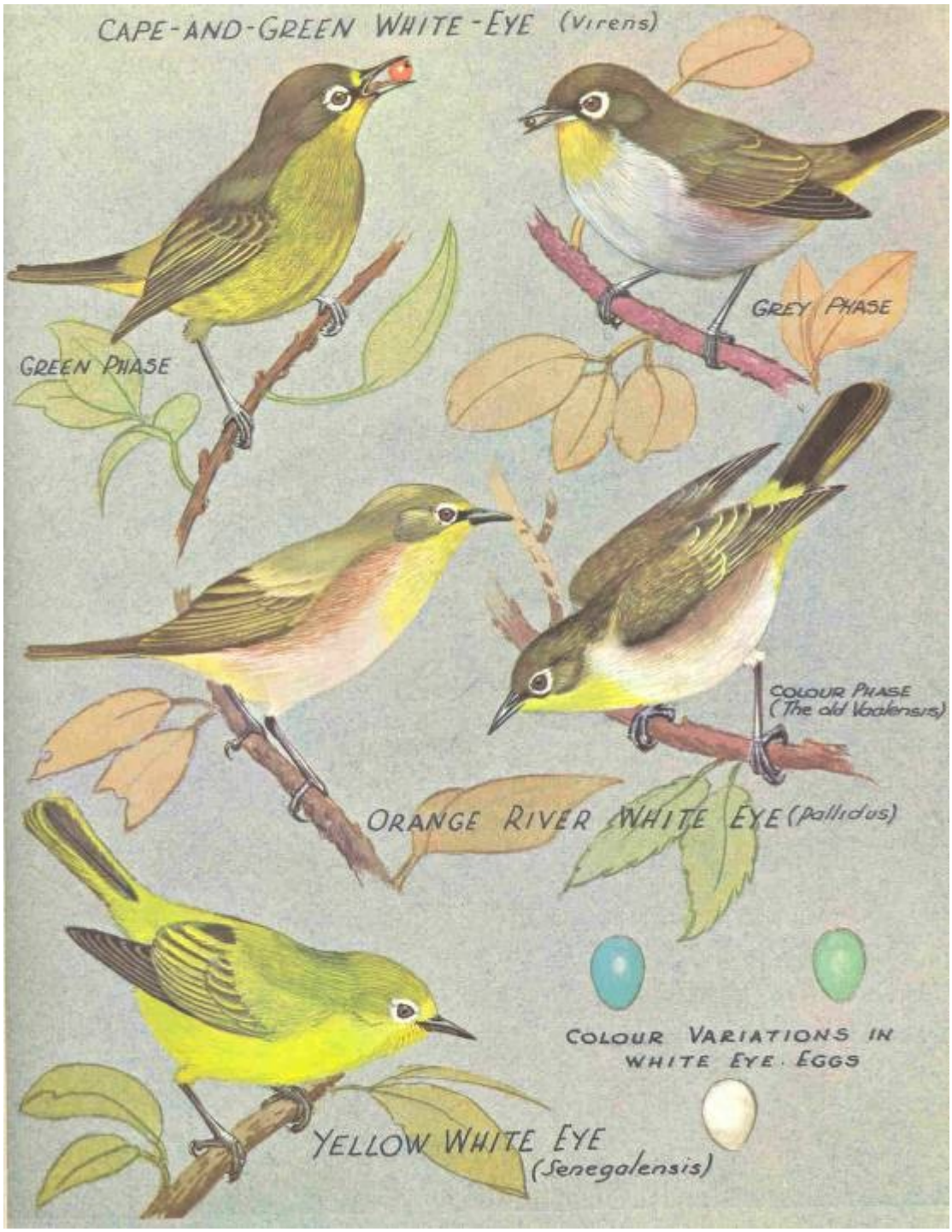

Frontispiece: Southern African *Zosterops* species and subspecies as recognized by, and taken from, Skead (1967).

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Abstract

White-eyes (Family Zosteropidae) are small passerine birds, with many species possessing a distinct ring of white feathers around the eye, while varying in underpart plumage colouration. There are currently 97 species within 13 genera placed in the Zosteropidae, occurring in Africa, Asia, Australia and Oceania. The most diverse genus, *Zosterops*, currently comprises 75 species which include all southern African white-eyes. At present, number of southern African *Zosterops* species recognized varies from one to three. Differing views have been adopted over the past 75 years, with taxa either being split according to underpart colouration or lumped together due to interbreeding between taxa.

The aim of this study was to incorporate all lines of evidence to establish the true taxonomy and phylogeny of southern African *Zosterops*. Character data sets used include plumage and morphometric measures, vocal characters and molecular (mitochondrial and nuclear) DNA sequences. A broad scale phylogeographic analysis was also performed to establish the evolutionary process driving the diversity observed among these birds.

Morphological characters used were those also available to past *Zosterops* taxonomists and included underpart plumage colouration and anatomical measurements (bill-, tarsus-, wing-, and tail-length). Characters were obtained from over 500 specimens and were analyzed using both uni- and multi-variate statistical methods. Morphometric measures on their own are fairly poor taxonomic indicators for southern African *Zosterops*. When used in combination with percentage underpart plumage colouration, however, four highly diagnosable southern African *Zosterops* Evolutionarily Significant Units (ESU) emerged: the Orange River White-eye *Z. pallidus*, the Grey Cape White-eye *Z. capensis*, the Green Cape White-eye *Z. virens* and the African Yellow White-eye *Z. senegalensis*.

Vocal data, both song and contact calls, was shown to be of taxonomic importance for southern African *Zosterops*. There was a clear distinction between *Z. pallidus* song and other

Zosterops song, while the contact calls of *Z. pallidus* and hybrid individuals were significantly different to those of *Z. capensis* and *Z. virens* individuals. Adaptive differences in vocal characters were likely to be habitat driven, with the arid-adapted *Z. pallidus* requiring longer vocalizations and more elements per song/call in order to communicate with conspecifics.

In order to establish a complete molecular phylogeny of southern African *Zosterops*, samples from well within the distribution range of the four ESU described using morphological techniques were sequenced for five molecular loci. One mitochondrial (ATP6), two nuclear (GAPDH and TGFβ2) and two sex-linked (CHD1 and MUSK) loci were used. Likelihood and Bayesian analysis on the ATP6 data set showed similar topologies. Surprisingly, *Z. senegalensis* was shown to be sister to a clade of *Z. capensis* and *Z. virens* individuals; *Z. pallidus* was basal to this clade. The total combined data set and species tree estimates produced similar results to the mtDNA data. Divergence time estimates show that southern African *Zosterops* diverged during the middle Pleistocene.

Mechanisms accounting for southern African *Zosterops* diversity were inferred by applying phylogeographic, and in particular coalescent analyses, in conjunction with biome and environmental data from taxon sampling localities. Although biomes did not account for much of the genetic diversity observed, environmental variables were shown to influence the distribution of the four *Zosterops* phenotypes of the region. This suggests that climate has had a recent influence on *Zosterops* diversity, an effect yet to be expressed in the neutral genetic data. Introgressive hybridization was found to occur from *Z. pallidus* into *Z. capensis* and *Z. virens*, specifically in areas of overlap between taxa. Although these taxa have been shown not to be sister, this secondary contact is likely to be facilitated by recent expansion of both *Z. capensis* and *Z. virens* into the range of *Z. pallidus*.

With the use of multiple lines of evidence, three species of southern African *Zosterops* could be recognized. They were: Orange River White-eye *Zosterops pallidus*, African Yellow White-eye *Zosterops senegalensis*, and Cape White-eye *Zosterops virens*, of which two subspecies are recognized: *Zosterops virens capensis* and *Zosterops virens virens*. Although these subspecies are morphologically distinct (underpart plumage colouration), similarities in vocalizations and DNA suggests that these taxa have yet to reach independent evolutionary trajectory that warrants species status.

Future prospects, specifically focusing on the nature of *Zosterops* hybrid zones and genes responsible for phenotypic divergence and speciation, are discussed.

Chapter 1

An overview of the taxonomy, phylogeny and eco-biogeography of southern African white-eyes (*Zosterops* spp.)

1.1. Introduction

1.1.1. White-eyes

White-eyes (Family Zosteropidae) are small, typically yellow and green passerine birds possessing a distinct ring of white feathers around the eye (Gill 1936, Roberts 1942, McLachlan & Liversidge 1978, Fry *et al.* 2000), hence their common name: white- or silver-eyes. White-eyes have a diverse diet, consisting mainly of fruit and nectar (Roberts 1942), and occasionally insects (Gill 1936, McLachlan & Liversidge 1978, Fry *et al.* 2000). Feeding is facilitated by a partially grooved tongue (Gill 1936, Roberts 1942, McLachlan & Liversidge 1978, Fry *et al.* 2000). A characteristic feature of white-eyes is that they have a rudimentary outermost primary feather, thus possessing only nine primaries (Roberts 1942, McLachlan & Liversidge 1978). Southern African white-eyes lay between three (Roberts 1942) and five (Gill 1936) pale blue eggs that are laid in a basin-shaped nest in early summer (Roberts 1942), with incubation lasting 11-12 days for both the Cape (*Zosterop virens*) and Orange River White-eyes (*Z. pallidus*) (*sensu* McLachlan & Liversidge 1978). The incubation period of the African Yellow White-eye (*Z. senegalensis*) has not been described. Young birds are often duller in colour than adults, with the white eye-ring forming when the young bird is about five weeks old (Frandsen 1982). White-eyes are often found in small parties that associate for much of the year (Gill 1936), and are usually found in woodland and forest edge; often only one *Zosterops* species is found locally (Hockey *et al.* 2005). Many mainland forms of *Zosterops* are often locally sedentary, although the clade clearly exhibits an extraordinary ability to colonize islands, promoting the formation of many endemic island forms (Hockey *et al.* 2005, Warren *et al.* 2006, Moyle *et al*. 2009, Melo *et al*. 2011).

1.1.2. Systematics

The systematics of the Zosteropidae has been contentious, involving the recognition of many putative often morphologically similar taxa, sometimes with complex geographical distributions (Fry *et al.* 2000). This is almost certainly due to the dispersal potential of *Zosterops* spp. (Warren *et al.* 2006, Moyle *et al*. 2009, Clegg and Phillimore 2010, Melo *et al*. 2011) that might undermine the effects of diversification in allopatry.

There are currently 97 species placed in the Zosteropidae within 13 genera, occurring in Africa, Asia, Australia and Oceania (Dutson 2008, van Balen 2008, Fjeldså *et al*. 2010). The white-eyes are most closely related to *Yuhina* and *Stachyris* babblers, members of the Timaliidae (van Balen 2008, Moyle *et al*. 2009). The most diverse genus, *Zosterops*, currently comprises 75 species which include all southern African white-eyes (Hockey *et al.* 2005, Dutson 2008, van Balen 2008), making it the most species-rich bird genus globally, now that *Nectarinia* has been 'deposed' from that position through the research of Rauri Bowie (2003) and others (Warren *et al.* 2003).

African *Zosterops* spp. appear to have been derived from a dispersal event from Asia (Warren *et al.* 2006, Moyle *et al*. 2009). At present the variation in the number of southern African *Zosterops* varies. In the most recent regional treatment, Hockey *et al.* (2005) recognize three species: *Z. virens* (Cape White-eye) (Smith & Bowie 2005), *Z. pallidus* (Orange River White-eye) (Bowie 2005) and *Z. senegalensis* (African Yellow White-eye) (Smith 2005). In contrast, the most recent global treatment of the Zosteropidae (van Balen 2008) a single species, *Z. pallidus*, comprising six subspecies (*Z. p. pallidus*, *Z. p. capensis*, *Z. p. caniviridis*, *Z. p. sundevalli*, *Z. p. virens* and *Z. p. atmorii*) is recognized.

At least two of these species (*Z. pallidus* and *Z. virens*) have been previously lumped into a single species (Roberts 1942, Clancey 1967, McLachlan & Liversidge 1978, Fry *et al.* 2000). Furthermore, numerous subspecies have been recognized within the various putative

species taxa (Roberts 1942, Clancey 1967). To date, these classifications have been based on variation in plumage colour (especially of the underparts), morphometrics, behaviour and ecology. Species/subspecies limits have been set based on putative hybridization (Clancey 1967). Below I review the taxonomic history of southern African Zosteropidae, and frame the primary questions that this thesis will seek to address.

1.2. Taxonomic history

1.2.1. Early days

Based on variation in plumage colour, Gill (1936) recognised five species of white-eyes within southern Africa (Table 1.1; Fig. 1.1 & 1.2).

1. Cape White-eye *Zosterops capensis* Sundervall (1850); grey flanks and belly (Fig. 1.1 & 1.2, i & ii) -- Western Cape to Port Elizabeth, Eastern Cape, north to Olifants River.

2. Green White-eye *Z. virens* Sundevall (1850); green flanks and belly (Fig. 1.1 & 1.2, iii & iv) -- eastern Eastern Cape, Kwa-Zulu Natal and north to Limpopo.

3. Orange River White-eye *Z. pallidus* Swainson (1838); cinnamon flanks and pale belly (Fig. 1.1 & 1.2, v - vii) -- Orange and Vaal Rivers and their tributaries, and Namibia.

4. Vaal River White-eye *Z. vaalensis* Gunning & Roberts (1911); brown flanks and green belly (Fig. 1.1 & 1.2, viii) -- Vaal River in Gauteng and northern Free State.

5. African Yellow White-eye *Z. senegalensis* Bonaparte (1850) [referred to by Gill (1936) as *Z. flavilateralis*]; yellow flank and belly (Fig. 1.1 & 1.2, ix) -- northeastern Kwa-Zulu Natal, Swaziland, Mozambique and Zimbabwe.

Roberts (1942) recognized four species of southern African white-eyes, including numerous subspecies (Table 1.1). The four species were: *Z. capensis,* Cape White-eye, comprising five subspecies (*Z. c. capensis* (Fig. 1.1 i), *Z. c. atmorii* (Fig. 1.1 ii), *Z. c. pallidus*

(Fig. 1.1 v) and *Z. c. deserticola* (Fig. 1.1 & 1.2, vi & vii), *Z. vaalensis* (Fig. 1.1 & 1.2, viii), *Z. senegalensis* (Fig. 1.1 & 1.2, ix) and *Z. virens* (Fig. 1.1 & 1.2, iii & iv).

1.2.2. Moreau

In what has been described as a classic publication (Mayr 1963), a comprehensive classification of African Zosteropidae by Moreau (1957) recognised three species of southern African white-eye: *Z. virens* (Fig. 1.1 & 1.2, i – iv), *Z. pallidus* (Fig. 1.1 & 1.2, v – vii) and *Z. senegalensis* (African Yellow White-eye) (Fig. 1.1 & 1.2, ix) which he referred to as *Z. anderssoni* (Table 1.1). These taxa were also delineated mainly on plumage colour, but he also considered other factors such as geography, ecology and morphometrics in his deliberations. Futhermore, Moreau (1957) divided southern African White-eyes into four belly/flank-colour categories: grey-, green-, pale- (currently often described as peachy) and yellow-bellied white-eyes.

Grey-bellied white-eyes: Moreau (1957) recognized two subspecies (Fig. 1.3) of the grey-bellied Cape White-eye, *Z. virens capensis* (Fig. 1.1 & 1.2, i) and *Z. v. atmorii* (Fig. 1.1 & 1.2, ii). The proposed transition zone between the two subspecies was thought to occur in a zone in the Eastern Cape that includes the towns of Murraysburg, Graaf Reinet and Port Elizabeth (Fig. 1.3). *Zosterops v. atmorii* (Fig. 1.1 & 1.2, ii) differs from *Z. v. capensis* by having more yellow on the throat, as well as having yellow on the forehead and lores. Northern populations of both *Z. v. capensis* and *Z. v. atmorii* have duller upperparts (Fig. 1.2, i & ii), possibly due to drier conditions found in these areas.

Green-bellied white-eyes: According to Moreau (1957), only one taxon of the green-bellied Cape White-eyes, within which there is considerable morphological variation (Fig. 1.3), warrants subspecies status: *Z. v. virens*. This subspecies is found in the Eastern Cape, Kwa-Zulu Natal, southern Gauteng and Mpumalanga (Fig. 1.3). It differs from the grey-

bellied forms in possessing a green belly (Fig. 1.1 & 1.2, iii & v). As in the grey-bellied forms, northern populations of green-bellied white-eyes appear to have generally duller upperparts than their coastal counterparts (compare Fig. 1.2, iii & v). This is, once again, thought to be due to drier conditions further north (Moreau 1957).

Pale-bellied white-eyes: These forms, found mainly in the Orange River Basin, were recognised as a monotypic species, the Orange River White-eye *Z. pallidus* by Moreau (1957; Fig. 1.3). In comparison to *Z. virens*, the Orange River White-eye (Fig. 1.1, v - vii) is generally paler yellow overall with grey-green upperparts, a generally white belly and cinnamon/peachy coloured flanks. There are also noteworthy differences between these two putative species in beak shape and, apparently, vocalizations (Moreau 1957). *Zosterops pallidus* (Fig. 1.1 & 1.2, v - vii) is partially sympatric with *Z. v. capensis* (Fig. 1.1 & 1.2, i) in the northern parts of the Western Cape, but seasonal movements of the two taxa may preclude sympatry during the breeding season (Moreau 1957).

Yellow-bellied white-eyes: The African Yellow White-eye *Zosterops senegalensis* (Fig. 1.1 & 1.2, ix) ranges from eastern Kwa-Zulu Natal and Mpumalanga, northwards into East Africa (Moreau 1957, Fig. 1.3). Although found in partial sympatry with *Z. v. virens* (Fig. 1.1 & 1.2, iii), there is an ecological separation between the two, with *Z. senegalensis* occurring in the deciduous bush of the low-veld, whereas the various green-bellied forms of *Z. virens* are found in the evergreen forests of coastal acacia (Moreau 1957). There are also other plumage and morphological differences between yellow-bellied and green-bellied white-eyes (Fig. 1.1 & 1.2, iii, iv & x). The yellow-bellied birds have a higher tail/wing ratio, and green-bellied birds have a far higher melanin content (plumage less yellow) on the feather barbs and barbules than in yellow-bellied forms (Moreau 1957).

To summarise, Moreau (1957) recognised three species of southern African white-eyes (Table 1.1). Within *Z. virens*, he recognised three subspecies, *Z. v. atmorii* (grey-

bellied), *Z. v. capensis* (grey-bellied) and *Z. v. virens* (green-bellied). The two other colour forms of white-eye (pale- and yellow-bellied) were afforded specific status, *Z. pallidus* and *Z. senegalensis*.

1.2.3. Skead

Skead (1967) followed Moreau (1957) in recognizing three species of white-eyes within southern Africa (Table 1.1), also splitting them into groups of subspecies based on colour of the plumage of the underparts. The green- and grey-bellied white-eyes were lumped into a single species *Z. virens* (Fig. 1.1 & 1.2, i - iv), comprising four [not three, as stated by Moreau (1957)] subspecies (Fig. 1.4): *Z. v. capensis* (Fig. 1.1 & 1.2, i), *Z. v. atmorii* (Fig. 1.1 & 1.2, ii), *Z. v. virens* (Fig. 1.1 & 1.2, iii) and *Z. v. caniviridis* (Fig. 1.1 & 1.2, iv). These subspecies differ primarily in flank colour. Both *Z. v. atmorii* and *Z. v. capensis* have grey flanks (Fig. 1.1, i & ii), whereas *Z. v. caniviridis* and *Z. v. virens* have green flanks (Fig. 1.1, iii & iv), with *Z. v. caniviridis* differing from *Z. v. virens* in being much greyer green on the upperparts (Fig. 1.2, iii & iv). Grey- and green-bellied birds were reported to have similar vocalizations (Skead 1967).

Within the Orange River White-eye *Z. pallidus*, Skead (1967) also differed from Moreau (1957) in recognising two subspecies, *Z. p. pallidus* (Fig. 1.1 & 1.2, vi & vii) and *Z. p. sundevalli* (Fig. 1.1 & 1.2,v; Fig. 1.4). *Zosterops p. pallidus* occurs along the western Orange River and into Namibia, whereas *Z. p. sundevalli* is found further east in the Vaal River Basin, the northeastern provinces of South Africa and the Free State (Fig. 1.4). *Z. p. sundevalli* is darker, and in general, less yellow than *Z. p. pallidus*, and its flanks are less cinnamon than in *Z. p. pallidus*. Although the general pattern of the Orange River White-eye's call was reported to be similar to that of the Cape White-eye, its tones are very

different; being more subdued, sometimes even muted, and in general somewhat deeper in tone (Skead 1967).

1.2.4. Clancey

Clancey (1967) attempted to build on Moreau's (1957) classification by adding data from specimen material from previously unsampled areas in southern Africa. Clancey (1967) recognised two species and four subspecies (Table 1.1): Grey White-eye *Z. pallidus capensis* (Fig. 1.1 & 1.2, i) and *Z. p. atmorii* (Fig. 1.1 & 1.2, ii); Green White-eye *Z. p. virens* (Fig. 1.1 & 1.2, iii & iv); Pale White-eye *Z. p. pallidus* (Fig. 1.1 & 1.2, v – vii); and the Yellow White-eye *Z. senegalensis* (Fig. 1.1 & 1.2, ix) that showed clinal variation in plumage colour.

The plumage of the Grey White-eye found in the western and southern interior areas of South Africa (Fig. 1.5) has yellow confined to the chin, with the fore-throat and under the tail-coverts being white to greyish-white (Fig. 1.1, i & ii). The Green White-eye from the eastern and northern areas have lemon to greenish-yellow underparts and light olive breast and flanks (Fig. 1.1, iii & iv). Near Garies in the Northern Cape (Fig. 1.5), the Grey White-eye comes into contact with the Pale White-eye, although two forms seemed to be parapatric with no interbreeding. Towards the east, *Z. p. capensis* (Fig. 1.1, i & ii) and *Z. p. virens* (Fig. 1.1, iii & iv), are primarily sympatric and appear to be reproductively compatible. The zone of contact between these two colour forms occurs along the drainage of the Buffalo River near East London (Fig. 1.5) up along the seaward face of the Drakensburg into the northeastern area of Kwa-Zulu Natal, ending near Fouriesburg and Harrismith in the southeastern Free State. Along this zone, mixed flocks are often found, with interbreeding between the grey- and green-bellied forms described in the King William's Town district.

Clancey (1967) also found a large amount of size variation among populations of the pale-bellied white-eyes. Populations from the west had shorter wings and bills compared to

eastern populations, whereas tail length was similar. As stated above, the pale-bellied and grey-bellied forms meet in the Northern Cape, without any apparent interbreeding. The pale-bellied birds, however, mix with the green-bellied forms in the northern Free State and possibly interbreed. The taxon, not recognised by Clancey (1967) at the time as a separate species, *Z. vaalensis* (Fig. 1.1 & 1.2, viii), appeared to possess characters that were intermediate between pale- and green-bellied white-eyes and this was considered to be a consequence of hybridization (Clancey 1967).

Clancey's (1967) classification (Table 1.1) treats the grey-, green- and pale-bellied birds as a polytypic species, *Z. pallidus*, based on interbreeding between putative pale- and grey-bellied birds with the green-bellied form, even though the pale- and grey-bellied forms are completely allopatric. Subspecies included within *Z. pallidus* are: *Z. p. pallidus* (Fig. 1.1 & 1.2, v – vii), *Z. p. capensis* (grey-bellied white-eyes, Fig. 1.1 & 1.2, i & ii) and *Z. p. virens* (green-bellied white-eyes; Fig. 1.1 & 1.2, iii & iv; Fig. 1.5). Clancey's (1967) classification became the standard in further publications on southern African *Zosterops* (McLachlan & Liversidge 1978, Monroe & Sibley 1993, Fry *et al.* 2000).

1.2.5. Status quo

Within the past five years, two separate opinions have been published regarding southern African *Zosterops* taxonomy. Following on from Moreau (1957) and Skead (1967), Hockey *et al*. (2005) recognizes three species.

1) the African Yellow White-eye, *Z. senegalensis*, comprising three subspecies: *Z. s. anderssoni* (Fig. 1.1 & 1.2, ix), *Z. s. stierlingi* and *Z. s. tongensis* (Smith 2005).

2) the Cape White-eye *Zosterops virens* (Fig. 1.1 & 1.2, i – iv) is subdivided into two subspecies: *Z. v. capensis* (including *Z. v. atmorii*; Fig. 1.1 & 1.2, i & ii) and *Z. v. virens* (including *Z. v. caniviridis*; Fig. 1.1 & 1.2, iii & iv; Smith & Bowie 2005).

3) the Orange River White-eye *Zosterops pallidus*. The absence of biogeographical breaks that could promote speciation, although minor differences in underpart colouration and size between birds suggest that *Z. pallidus* is polytypic with three subspecies (Bowie 2005): *Zosterops p. sundevalli* (Fig. 1.1 & 1.2, v), upper Orange River as well as the lower and middle reaches of the Vaal River, *Zosterops p. deserticola* (Fig. 1.1 & 1.2, vi) is found in the lower Orange River, *Z. p. haigamchabensis* (Fig. 1.1 & 1.2, vii), western central Namibia south towards the Northern Cape province of South Africa (Bowie 2005).

The most recent treatment of southern African *Zosterops* taxonomy (van Balen 2008) takes on the other end of the taxonomic spectrum, adhering closely with the classification proposed by Clancey (1967). Six subspecies are recognized under this classification:

1) *Zosterops pallidus pallidus* – Namibia and northwestern South Africa
2) *Zosterops pallidus capensis* – southwestern South Africa
3) *Zosterops pallidus caniviridis* – east and southeastern Botswana, and northern South Africa
4) *Zosterops pallidus sundevalli* – central South Africa
5) *Zosterops pallidus virens* – southwestern Mozambique, Swaziland and east South Africa
6) *Zosterops pallidus atmorii* – Lesotho and adjacent South Africa

1.3. Summary

Taking the extreme 'splitters' view, based largely on analyses of the colour of underpart plumage and morphometrics, nine species/subspecies of *Zosterops* have been recognized within southern Africa (Fig. 1.6). These are regarded as taxonomic hypotheses and their validity is assessed in Chapter 2.

1.4. Hypotheses to be investigated

1) *Is there any morphological and genetic spatial pattern of structure within southern African* Zosterops *spp.?*

Null Hypothesis: There is no congruent morphological and genetic spatial pattern within southern African *Zosterops* spp.

Alternative hypothesis: There is congruent morphological and genetic spatial pattern within southern African *Zosterops* spp.

Predictions: Within the green-bellied Cape White-eye *Z. v. virens*, two plumage types are predicted to occur. A bright green, coastal form from the Eastern Cape and KwaZulu-Natal, and a dull green, highland form (referred to in previous classifications as a separate subspecies *Z. v. caniviridis*). The Drakensburg Escarpment will act as a barrier to gene flow, resulting in a phylogeographic break between the coastal, lowland and highland populations.

Following Moreau 1957, the grey-bellied Cape White-eye *Z. v. capensis* is thought to comprise two plumage types. Individuals with uniformly grey-bellies are found along the coast and interior of the Western Cape and parts of the Eastern Cape ending along a line formed by Murraysburg, Graaf Reinet and Port Elizabeth (Moreau 1957). The second colour form (referred to in previous classifications as a separate subspecies as *Z. v. atmorii*) has a partially grey belly mixed with white, and possesses bright yellow lores, not found in the western form (Skead 1967). The genetic structure of the grey-bellied Cape White-eye is thought to match that of the plumage, where a switch in rainfall seasonality, from west to east, is expected to affect gene flow between western and eastern populations of *Z. v. capensis*.

The cinnamon-flanked Orange River White-eye *Z. pallidus* does not show any plumage variation among any of its subspecies; however, there is a large amount of size variation among populations. Populations from the west (putative subspecies *Z. p. deserticola*

and *Z. p. haigamchabensis*) had shorter wings and bills compared to eastern populations (*Z. p. sundevalli*) (Clancey 1967). Results of DNA analyses are expected to be congruent with changes in size observed in these taxa unless selection has been very strong, a possibility given the heterogeneous nature of southern Africa (see e.g. Ribeiro *et al*. 2011).

2) *If there is spatial genetic structure, do phylogeographic breaks correspond to changes in environmental features?*

Null hypothesis: Phylogeographic breaks do not correspond to changes in environmental features.

Alternative hypothesis: Phylogeographic breaks do correspond to changes in environmental features.

Predictions: In the Cape White-eye *Z. virens*, the main break is expected to occur at the boundary between subspecies, in the Eastern Cape region, where increases in humidity and a change from a winter to a summer rainfall regime occur. In this region, there is also a change in habitat structure, from predominantly Fynbos habitat, through to Thicket and then Savanna further towards the east. The Eastern Escarpment is expected to restrict gene flow between highland and lowland *Z. v. virens* populations, whereas in the west, genetic breaks are expected to occur at the boundary between winter and summer rainfall regions, located in the southern Cape.

In the Orange River White-eye *Z. pallidus*, due to the extreme aridity of its habitat, the sparseness of its preferred habitat and changes in rainfall from west to east, genetic breaks are expected to be similar to that of other arid adapted southern African taxa, occurring in the Knersvlakte region of the Northern Cape (Matthee and Robinson 1996, Matthee and Flemming 2002, Smit *et al*. 2007, 2010, Portik *et al*. 2011).

3) *Have past climate changes (Plio-Pleistocene) shaped genetic variability in southern African* Zosterops*?*

Null hypothesis: Past climate changes (Plio-Pleistocene) have not shaped genetic variability in southern African *Zosterops*.

Alternative hypothesis: Past climate changes (Plio-Pleistocene) have shaped genetic variability in southern African *Zosterops*.

Predictions: All southern African white-eye taxa are arboreal, moving between trees in search of food, and are therefore not restricted to any one vegetation type or biome. In the east, *Z. v. virens* occupies forest (coastal and montane), bushy kloof, riverine scrub and savanna habitats, whereas *Z. v. capensis* in the west is found in Karoo and Fynbos shrublands, as well as mountain forest (Smith & Bowie 2005). Restricted to the river courses of the Orange and Vaal Rivers, *Z. pallidus* is found mainly in the large trees and thorn scrub associated with these river courses (Bowie 2005). The African Yellow White-eye (*Z. senegalensis*) occurs predominately in mixed woodland with grassy understory (savanna) and, in places, evergreen forest (Smith 2005).

Although not completely restricted by the decrease in forest environment during Pleistocene glaciations (van Zinderen Bakker 1978), the presence of savanna habitat in the east of southern Africa would have provided suitable habitat for a fairly widespread distribution of *Z. virens* and *Z. senegalensis* populations. The expansion of savanna and woodland vegetation during the wetter and warmer interglacial (van Zinderen Bakker 1978) would be associated with an expansion of Cape (west and south into South Africa) and African Yellow White-eyes (north and west into the rest of Africa). Further expansion of the Cape White-eye into the Western Cape would have been facilitated by the invasion of Karoo vegetation onto the Cape coastal plain (van Zinderen Bakker 1978).

The river-restricted Orange River White-eye is expected to have closely followed the water courses during the dry, glacial period. Changes in the course of the Orange River would have dictated the distribution of this taxon during this period and may even have created breaks in the distribution which may possibly have caused genetic differences among populations.

It should be mentioned however, that the current ecological associations exhibited by southern African *Zosterops* may have changed over time.

4) *How have southern African* Zosterops *spp. responded to environmental change during the Holocene?*

There was a general wetter and warmer climate in southern Africa during the Holocene period (Tyson 1986). This would have facilitated the expansion of both Forest and Savanna biomes facilitating further expansion of *Z. virens* and *Z. senegalensis*. There is no predicted range restriction for these two species; therefore a stable effective population size is expected for these taxa.

I expect that the increase in rainfall post last glacial maximum (LGM) would have increased suitable habitat along river courses, allowing for the expansion of *Z. pallidus* out of the water-restricted refugia experienced during colder and drier periods (Tyson 1986), leading to a predicted population expansion in *Z. pallidus*.

5) *Do subspecies of* Z. pallidus *and* Z. virens *form monophyletic groups?*

Null hypothesis: The subspecies of *Z. pallidus* and *Z. virens* do not form monophyletic groups.

Alternative hypothesis: The subspecies of *Z. pallidus* and *Z. virens* do form monophyletic groups.

Predictions: There is fairly strong morphological diversity (plumage colouration) within *Z. virens* with up to five subspecies being described (Roberts 1942). Although at present only two subspecies are recognized (Smith & Bowie 2005), four distinct plumage groups are predicted, and as a result four monophyletic groups within *Z. virens*.

Differences in Orange River White-eye populations relate to size with subtle difference in the shade of plumage (Clancey 1967). Hence, with no distinct differences in plumage among *Z. pallidus* populations, and with changes in size possibly due to local adaptation, no monophyletic groups are expected to be found within *Z. pallidus*.

Tables

Table 1.1: Taxonomic name changes of the grey-bellied Cape White-eye, green-bellied Cape White-eye, Orange River White-eye and African Yellow White-eye.

Author	Grey Cape White-eye	Green Cape White-eye	Orange River White-eye	African Yellow White-eye	Additional taxa
Gill (1936)	*Z. capensis*	*Z. virens*	*Z. pallidus*	*Z. flaviventris*	*Z. vaalensis*
Roberts (1942)	*Z. capensis* (5 subspecies: *Z. c. capensis, Z. c. atmorii, Z. c. basuticus, Z. c. pallidus, Z. c. deserticola)*	*Z. virens*	*Z. c. pallidus*	*Z. senegalensis*	*Z. vaalensis*
Moreau (1957)	*Z. v. capensis* & *Z. v. atmorii*	*Z. v. virens*	*Z. pallidus*	*Z. andersonii*	
Skead (1967)	*Z. v. capensis* & *Z. v. atmorii*	*Z. v. virens* & *Z. v. caniviridis*	*Z. p. pallidus* & *Z. p. sundevalli*	*Z. senegalensis*	
Clancey (1967)	*Z. p. capensis*	*Z. p. virens*	*Z. p. pallidus*	*Z. senegalensis*	
Hockey *et al*. (2005)	*Z. v. capensis* (incl. *Z. v.atmorii*)	*Z. v. virens* (incl. *Z. v. caniviridis*)	*Z. p. sundevalli, Z. p. deserticola* & *Z. p. haigamchabensis*	*Z. s. andersoni*	
van Balen (2008)	*Z. p. capensis* & *Z. p. atmorii*	*Z. p. virens* & *Z. p. caniviridis*	*Z. p. pallidus* & *Z. p. sundevalli*	*Z. s. andersoni*	

Figure legends

Figure 1.1: Ventral view of putative species/subspecies taxa of *Zosterops* from southern Africa.

Figure 1.2: Dorsal view of putative species/subspecies taxa of *Zosterops* from southern Africa.

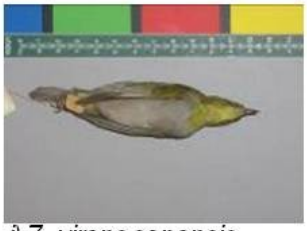
Figure 1.3: The distribution of white-eyes within southern Africa according to Moreau (1957). Three species are recognized: *Zosterops pallidus* (pale-bellied), *Z. virens* (grey- and green-bellied) and *Z. anderssoni* (yellow-bellied). Three subspecies are attributed to *Z. virens* (*capensis, atmorii* and *virens*).

Figure 1.4: The distribution of the grey- and green-bellied Cape White-eye (*Zosterops virens*) and the Orange River White-eye (*Zosterops pallidus*) in southern Africa according to Skead (1967). Four subspecies of the grey- and green-bellied Cape White-eye and two subspecies of the Orange River White-eye are recognized.

Figure 1.5: The distribution of white-eyes in southern Africa according to Clancey (1967). Two species are recognized, *Z. pallidus* and *Z. senegalensis* (not indicated on the map). Within *Z. pallidus*, three subspecies are recognized, *Z. p. pallidus*, *Z. p. capensis* and *Z. p. virens*. The other putative subspecies (*atmorii, caniviridis* and *sundevalli*) lack sufficient variation to warrant subspecies status.

Figure 1.6: *Zosterops* taxa recognized by Gill (1936), Roberts (1942), Moreau (1957), Skead (1967) and Clancey (1967).

Fig. 1.1



i) Z. virens capensis

ii) Z. virens atmorii

iii) Z. virens virens

iv) Z. virens caniviridis

v) Z. pallidus sundevalli

vi) Z. p. deserticola

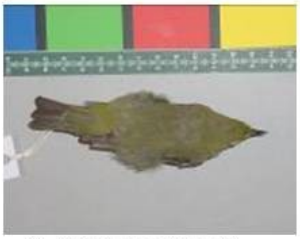
vii) Z. p. haigamchabensis

viii) Z. vaalensis

ix) Z. senegalensis

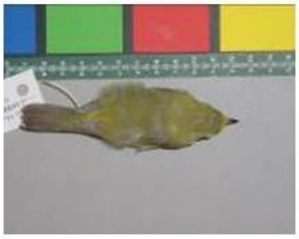
Fig. 1.2

i) Z. virens capensis



ii) Z. virens atmorii

iii) Z. virens virens



iv) Z. virens caniviridis

v) Z. pallidus sundevalli

vi) Z. p. deserticola

vii) Z. p. haigamchabensis

viii) Z. vaalensis

ix) Z. senegalensis

Fig. 1.3

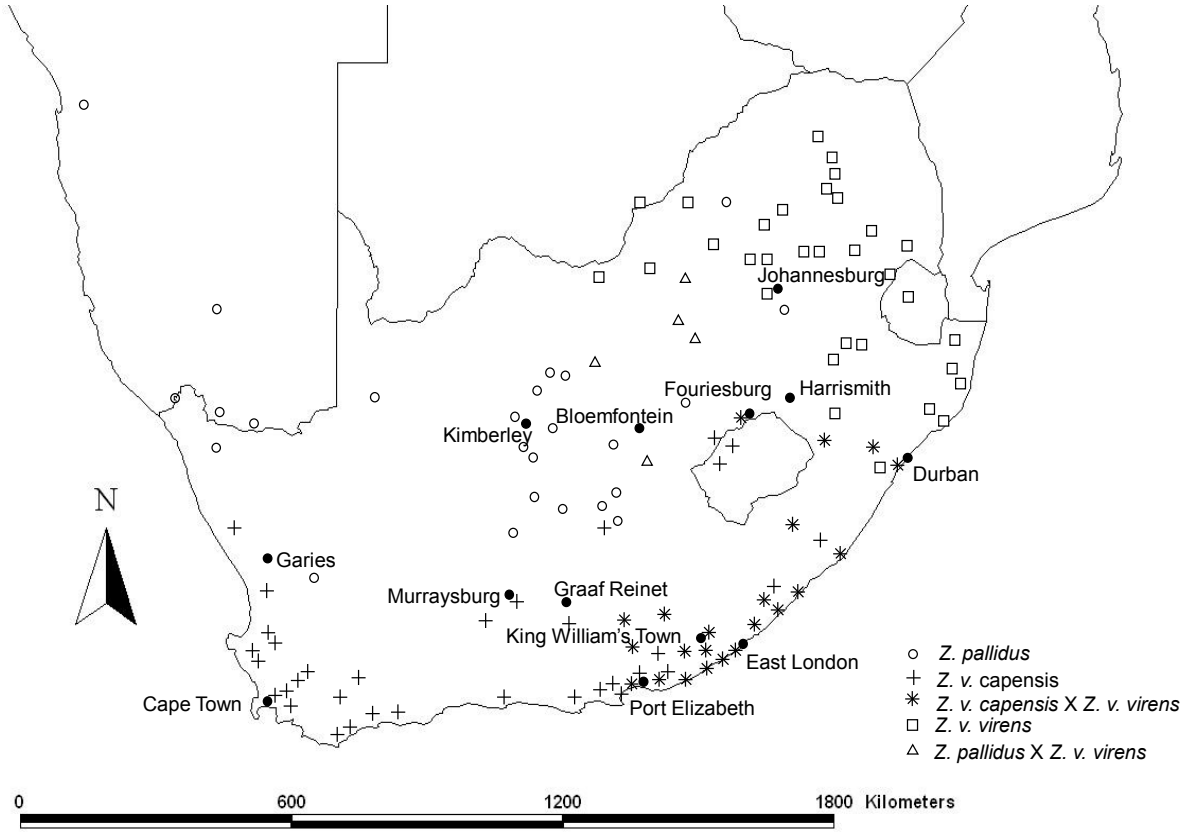


Fig. 1.4

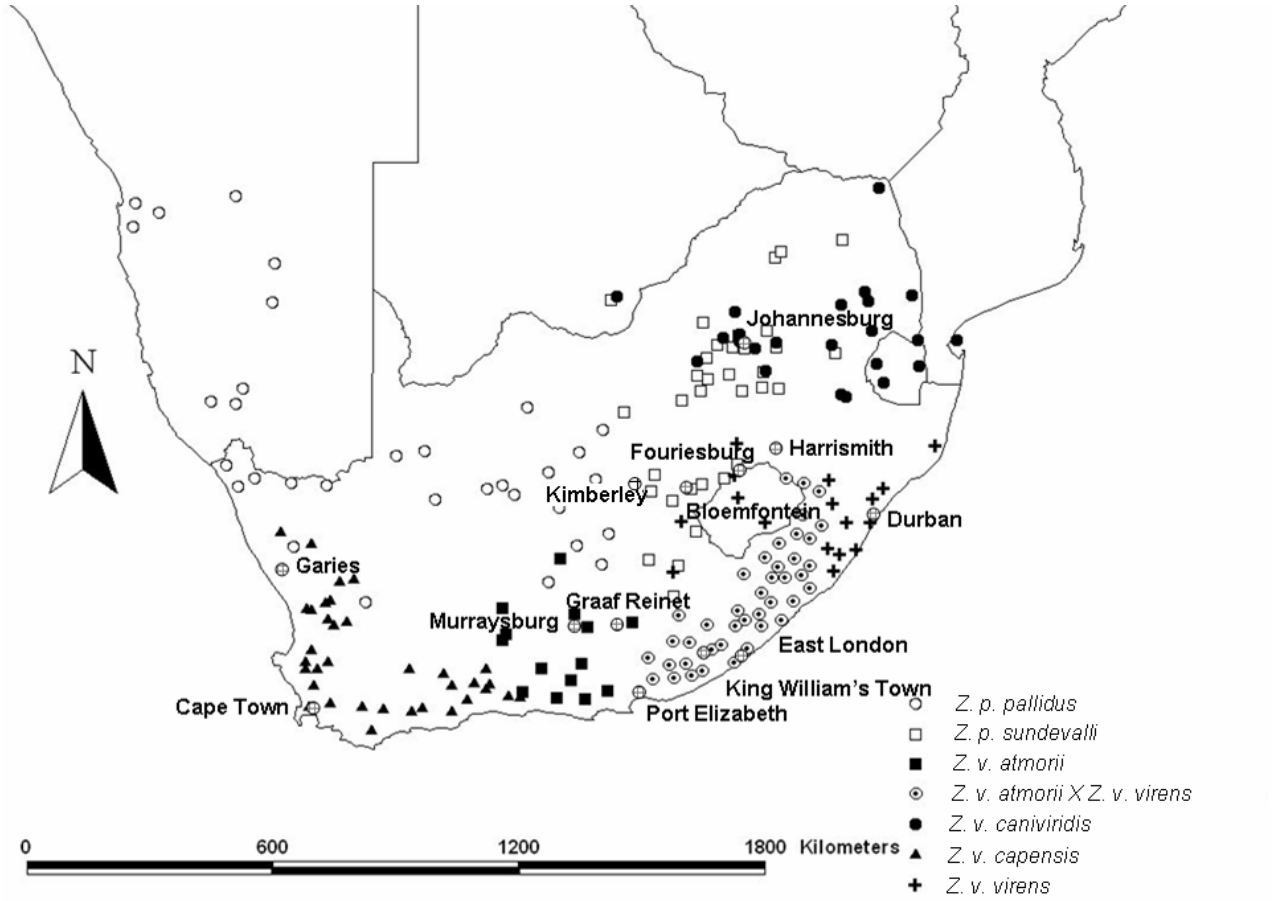


Fig. 1.5

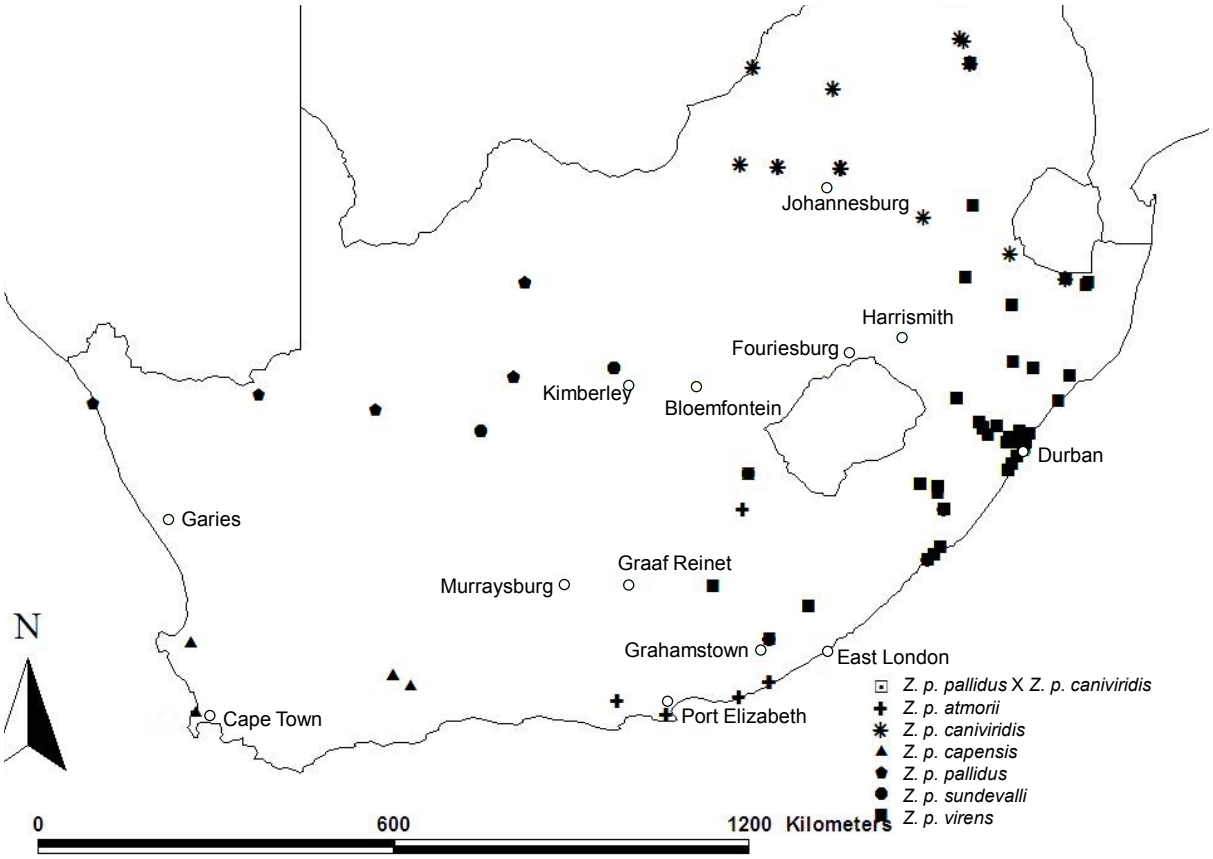
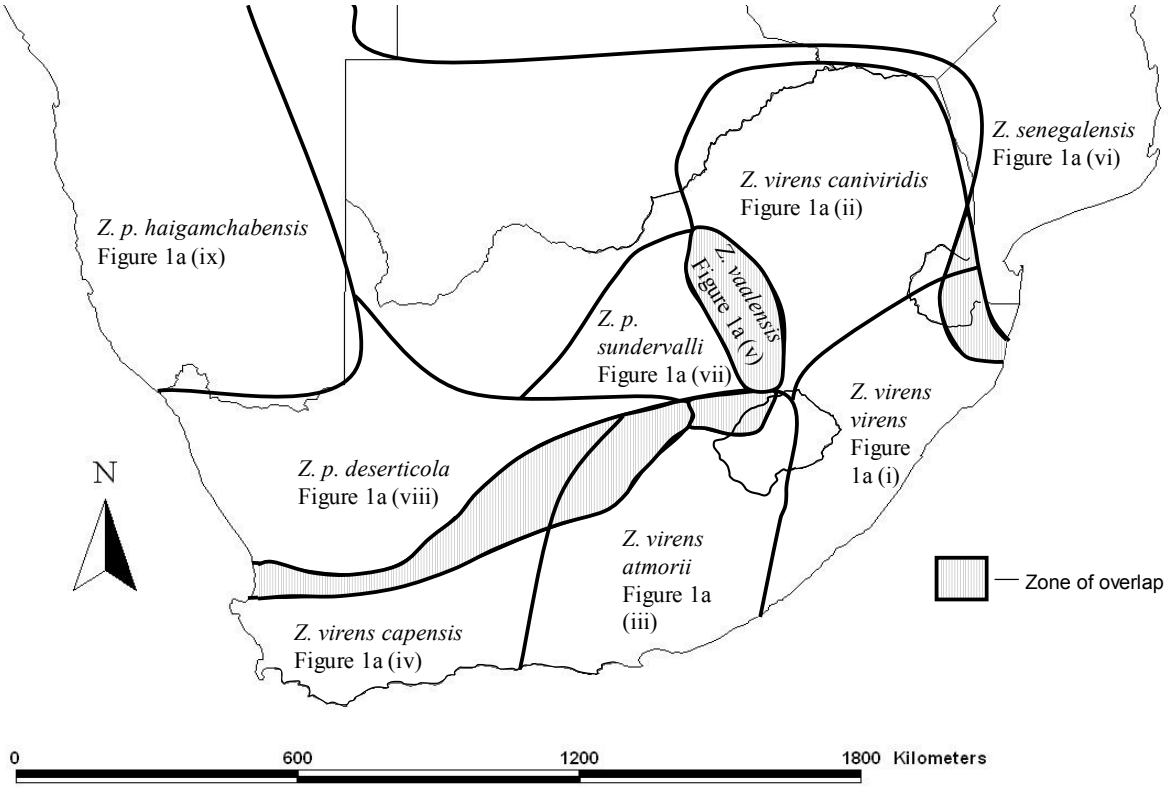


Fig. 1.6



Chapter 2

The use of subspecies in the systematics of southern African white-eyes: historical entities or eco-geographic variants

2.1. Abstract

The recognition of objectively diagnosable and evolutionarily significant terminal taxa, i.e. Evolutionarily Significant Units (ESU), is essential for the generation of defensible taxic hypotheses necessary for all forms of evolutionary and comparative biology and for effective guiding of biodiversity conservation. However, there has been a long and on-going, sometimes heated debate, on the merits of the subspecies category in this endeavour. To determine possible ESU present in southern African white-eyes, *Zosterops* spp., uni- and multi-variate statistical approaches were used to re-investigate the morphological characteristics (morphometric and plumage colouration) employed in past taxonomic studies to propose nine putative southern African *Zosterops* ESU, described at the time as subspecies. Four ESU emerged from these analyses. Geographical, discriminatory, multifaceted analyses suggest that these four taxa, *Z. senegalensis, Z. virens, Z. capensis* and *Z. pallidus* warrant species status.

2.2. Introduction

The use of modern statistical analysis to assess the congruence and conflict among gross organismal (e.g. behaviour and ecology), morphometric (discrete and continuous meristic characters) and molecular biological data has changed the face of taxonomy irrevocably. At one extreme there have been calls for DNA based methodology to have precedence (Tautz *et al.* 2002, 2003 Herbert *et al.* 2003, Mallet and Willmott 2003), although a multifaceted approach using a range of molecular and organismal characters as evidence has also received increased support (Hillis 1987, Crowe 1999, Will and Rubinoff 2004, Dayrat 2005, Alström *et al*. 2007, Alström *et al*. 2008), particularly when delimiting species or subspecies (Sites and Marshall 2004, Pruett and Winker 2010, Schlick-Steiner *et al*. 2010). In the past, the sole use of morphological evidence in delineating species and subspecies boundaries has had numerous problems, arising from the effects of, for example: sexual dimorphism, morphological variation among age classes, plumage or size polymorphisms and local variation within and among allopatric populations/taxa (Mayr *et al*. 1953, Wilson and Brown 1953, Cain 1954, Zink 2004). In particular, the utility of the continued use of subspecies, i.e. operational taxonomic units that differ markedly from each other in core parts of their distribution, but show varying degrees of intergradation in areas of para/sympatry (Cain 1954, Crowe 1978, Johnson *et al*. 1999, Dayrat 2005) has received considerable debate (e.g. Wilson and Brown 1953, Mayr 1982, Zink 2004, Phillimore and Owens 2006).

Molecular evidence has been used to adjudicate the status of subspecies that have been delineated traditionally from phenotypic characters alone. For example, this approach revealed the startling statistic that only 3% of continental Palearctic and Nearctic (areas with low levels of genetic differentiation) subspecies are discernable phylogenetic entities (Zink 2004). This however, may not be a general trend, as many island and continental subspecies

from other biogeographic realms have been shown to be phylogenetically distinct entities (Phillimore and Owens 2006).

Partly to avoid the debate centering on the criteria with which to delineate species and subspecies, Evolutionarily Significant Units **(**ESU) as first described by Ryder (1986), were used to recognise taxa based on the congruence of multiple sources of information. For birds, congruence could occur among: plumage, morphometrics, behaviour, ecology, range and distribution, and molecular data sets. Moritz (1994) added that the primary focus of ESU should be directed at historical population structure (e.g. based on reciprocal monophyly) and, in particular, historically isolated populations that possess a putatively higher evolutionary potential than other such populations.

In this chapter, morphological attributes that have been employed traditionally in the taxonomy of southern African white-eyes, *Zosterops* spp. to identify and rank ESU are analysed. Historically, avian subspecies were described if differences in plumage colouration or size occurred, where 75% of a population differed from other populations of the same species (Amadon 1949, Gill 1989), or more recently at a 95% level of diagnosability (Patten and Unitt 2002, Remsen 2010). This percentage cut-off has lead to some authors claiming that avian subspecies are often based on subjective, noncongruent changes (but see Remsen 2010, Winker 2010) in morphological characters (Zink 2004) and thus diminish the ability to recognize meaningful ESU. As a consequence many subspecies have come to represent interbreeding populations that are genetically (Smith *et al*. 1997) and/or phenotypically (Winker *et al.* 2007) discernable and should at least be described by one diagnosable morphological character state (Rising 2007, Remsen 2010, Winker 2010).

The systematics of the white-eyes (Zosteropidae) has been contentious, involving the recognition of many putative, often morphologically similar taxa, sometimes with complex geographical distributions (Fry *et al*. 2000, van Balen 2008). This is almost certainly due to

the dispersal potential of *Zosterops* species (Warren *et al.* 2006, Moyle *et al.* 2009, Melo *et al*. 2011, although see Clegg and Phillimore 2010) that might undermine the effects of diversification in allopatry.

African *Zosterops* species appear to have been derived from a dispersal event from Asia (Warren *et al.* 2006, Moyle *et al.* 2009, Melo *et al*. 2011). In African white-eyes, plumage differentiation is highly variable geographically, particularly in the colouration of the underparts, especially those of the flanks and the belly (Moreau 1957, van Balen 2008). Plumage colour of the underparts of southern African white-eyes varies in combination between white, cinnamon, grey, green and yellow (Gill 1936, Roberts 1942). The association of the various plumage morphs with specific biomes in southern Africa may indicate that the diverse nature of the underpart colouration of *Zosterops* taxa has arisen due to clinal or eco-geographic variation. As a consequence, the taxonomy of the southern African Zosteropidae has varied considerably over the past 70 years (Table 2.1). The aim of this chapter is to provide a hypothesis for the number of defensible terminal taxa of *Zosterops* within southern Africa through the quantitative analyses of morphological information which previous authors used to identify putative taxa and clinal variation (Gill 1936, Roberts 1942, Moreau 1957, Clancey 1967, Skead 1967). The aim is to diagnose taxonomic units or historical entities as opposed to clinal or eco-geographic variants with respect to the environmental heterogeneity represented by the diverse biomes of southern Africa.

2.3. Methods

2.3.1. *Taxon sampling*

Populations throughout the distribution of southern African *Zosterops* taxa, as well as *Z. senegalensis* populations from outside of southern Africa were sampled. Specimens representing nine of the most commonly recognized species and subspecies (Table 2.1, Fig.

2.1) were examined. These taxa are: *Zosterops senegalensis*, *Z. virens caniviridis*, *Z. v. virens*, *Z. v. atmorii*, *Z. v. capensis*, *Z. pallidus deserticola*, *Z. p. haigamchabensis, Z. p. sundevalli* and *Z. vaalensis*.

2.3.2. *Characters*

Morphological characters investigated included standard avian measurements: bill- (tip to feather line), tarsus-, tail- (insertion point on the pygostyle to the tip of the longest feather) and wing-length (flattened wing-chord), and scores for underpart colouration. Vernier callipers were used to take bill and tarsus measurements, and a wing and tail rule for the other measures. To assess variation in underpart plumage colouration within and between populations, a quantitative colour analysis was performed on 558 museum specimens representing all the above-mentioned taxa (see Fig. 2.1 for localities). Percentage colour scores were estimated using a digital grid superimposed over photographs of all the specimens, with the number of grid squares, or part thereof, counted for each of the five colours (grey, green, cinnamon, yellow and white) found on the underside (throat, belly, flanks and vent) of *Zosterops* individuals was estimated. To minimize the subjectivity of plumage colour assessment, digital photographs of 514 museum study skins were also taken under standardised lighting conditions from October 2006 – April 2007, using a Sony Cyber-shot DSC-H2 digital camera. Red, green and blue quantitative colour scores were obtained using the colour picker function in Adobe® Photoshop® 7.0.1 (Adobe Systems, Inc., San Jose, CA, USA) (Dale 2000). Fresh specimens collected during the course of this study were prepared and dried (> 6 months) to account for shrinkage prior to taking measurements and photographs.

2.3.3. *Statistical analyses*

Differences between the sexes of the nine putative taxa were tested using two-tailed *t*-test on log (x + 1) transformed data, although no discernable morphological differences between the sexes has been noted in the past (Skead 1967, Hockey *et al*. 2005). Differences in plumage colouration characters between taxa were tested using one-way ANOVA with a Newman-Keuls test to determine which taxa grouped together. As morphological measurements are not considered to be independent, a MANCOVA was used to test for overall differences in morphometric characters among the putative *Zosterops* taxa with tarsus-length as covariate and bill-, wing- and tail-length the dependant variables. Given the significant MANCOVA results, further morphometric analyses were conducted on data that was standardized for size by using tarsus-length.

All specimens were also analyzed using individual assignment/clustering methods without being classified *a priori* by taxon. Use was made of all quantitative measurements and assessments in combination and separately using PRIMER 6.1.5 (Plymouth Marine Laboratory, U.K.) to discover patterns of overall similarity among them. To identify patterns of hierarchical similarity, Euclidean distance was chosen as the similarity coefficient and multi-dimensional scaling analyses were performed for the four data sets: morphometrics, quantitative colour scores, percentage colour scores and combined morphometric and percentage colour scores.

2.3.4. *Assumed taxa as 'hypotheses'*

Means and standard deviations were obtained for each of the colour assessments and were graphed by putative taxon using Statistica 9 (Stat Soft, Inc. 2004). Given significant morphometric MANCOVA results, an ANCOVA was used for each variable independently to determine which variable differed among taxa. When confidence intervals for a

measurement or assessment for a taxon or group of putative taxa did not overlap with those for other putative taxa, that quantitative measurement or assessment was recorded as a bi- or multi-state qualitative character. Using these unordered, 'qualitative' characters, parsimony trees were constructed from four data sets: 1) morphometric measurements, 2) percentage colour scores, 3) quantitative colour scores and, 4) a combination of the measurement and percentage colour scores. These trees were constructed using the 'traditional method' (Wagner trees with TBR - branch-swapping, 100 replicates, saving 100 trees per replicate) using these discrete characters in TNT (Goloboff *et al.* 2003). In addition, continuous data were also analysed using methods developed by Goloboff *et al*. (2006). Bootstrap values were calculated using 1000 pseudo-replicates.

To determine the measurements/plumage assessments most useful in diagnosing the putative groupings, *a posteriori* discriminant function analyses were performed using Statistica 7 (StatSoft, Inc. 2004).

2.4. Results

No significant difference was observed between the sexes for any of the quantified characters within any of the nine putative *Zosterops* taxa.

2.4.1. *Morphometric characters*

Significant differences in morphometrics occurred among *Zosterops* taxa (MANCOVA: Wilks $\lambda = 0.266$, $P < 0.0001$), with the uni-variate ANCOVA showing differences in bill-, wing- and tail-length among all taxa (Table 2.2, Fig. 2.2). The MDS plot (Fig. 2.3a) representing *Zosterops* morphospace suggests that *Z. senegalensis* forms a group separate from the rest of the *Zosterops* taxa. *Zosterops senegalensis* individuals have longer bills and shorter wings and tails than most of the other *Zosterops* taxa (Table 2.2). The

discriminant function analysis confirmed that the morphometric data was able to discriminate > 90% of *Z. senegalensis* specimens from those of other *Zosterops* taxa with the misclassified individuals clustering with *Z. v. virens* (Table 2.3). The morphometric was also able to discriminate 93.5% of *Z. v. virens* specimens.

2.4.2. *Plumage colour scores*

Some distinct grouping is observed in the MDS analysis of quantitative colour scores (Fig. 2.3b) with three groups of *Zosterops* taxa discernable. From left to right, the first grouping follows the morphometric results, grouping the yellow *Z. senegalensis* individuals together. This group is very close to the second group which is mostly comprised of the green-bellied birds (*Z. v. virens* and *Z. v. caniviridis*). The grey-bellied birds (*Z. v. atmorii* and *Z. v. capensis*) group together with the Orange River White-eye individuals (*Z. p. sundevalli*, *Z. p. deserticola* and *Z. p. haigamchabensis*) in a third group. Birds of intermediate plumage colouration in the multidimensional morphospace are mainly found on the periphery between the grey-bellied and Orange River group, and the green-bellied group. A total of 79.2% of the 515 individuals clustered 'correctly', with *Z. senegalensis* and *Z. v. virens* being observed 100% (3 individuals) and 93.9% (232 individuals) correctly, respectively (Table 2.4). Other taxa that were also generally diagnosable were: *Z. v. atmorii*, *Z. v. capensis*, *Z. vaalensis, Z. p. deserticola* and *Z. p. haigamchabensis* (all > 70%; Table 2.4). The percentage colour score MDS analysis (Fig. 2.3c) recovered four groups from left to right. The first group is made up of green-bellied birds (*Z. v. virens* and *Z. v. caniviridis*) plus some *Z. senegalensis*. The second group is composed entirely of yellow-bellied birds. All grey-bellied birds (*Z. v. atmorii* and *Z. v. capensis*) form the third group and the fourth group is comprised of Orange River White-eyes (cinnamon flanked birds). Intermediate individuals are found within both the Orange River and green-bellied groupings. *Zosterops senegalensis*

was poorly diagnosed (41.3%; Table 2.5) in the discriminant function analysis of the percentage colour data set. *Zosterops v. virens*, *Z. v. capensis* and *Z. p. deserticola* were readily diagnosed (all > 80%; Table 2.5).

2.4.3. *Combined data*

The MDS analysis (Fig. 2.3d) of the combined data set (morphometric and percentage colour scores) is very similar to that of the percentage colour MDS analysis (Fig. 2.3c). Closer inspection of the green-bellied grouping reveals the presence of many intermixed *Z. senegalensis* individuals (Fig. 2.4) that belong to subspecies that are described as being greener than the nominate *Z. s. senegalensis* (Fry *et al*. 2000, van Balen 2008). The discriminant function analysis of the combined data set indicate that bill-length ($P < 0.001$; Partial Lambda = 0.62) is the primary discriminating factor that separates the nine southern African *Zosterops* taxa. Wing-length ($P < 0.001$; Partial Lambda = 0.74) and percentage grey underparts ($P < 0.001$; Partial Lambda = 0.89) are also contributing factors. In total, 75.3% of all 558 individuals were correctly classified (Table 2.6). *Zosterops v. virens* was diagnosed 99.6% correctly and the African Yellow White-eye *Z. senegalensis*, was diagnosed 89.1% correctly. The most poorly diagnosed taxon was *Z. v. caniviridis* at only 2.5%, with incorrectly classified birds being described as the geographically adjacent *Z. v. virens*. Of the grey-bellied birds, *Z. v. capensis* and *Z. v. atmorii* were diagnosed 80.4% and 50% correctly. All incorrectly classified *Z. v. atmorii* individuals were recognized as *Z. v. capensis*. Individuals that were diagnosed correctly were found to have more white on the belly than *Z. v. capensis*. Within the *Z. pallidus* putative subspecies, *Z. p. deserticola* was diagnosed 79.1% correctly (Table 2.6). *Zosterops vaalensis* was diagnosed 42.9% correctly; incorrectly described birds were split between *Z. v. virens* and the three *Z. pallidus* subspecies.

2.4.4. *Univariate and phylogenetic analyses*

Single most parsimonious trees were obtained from analyses of both the continuous and discrete morphometric data (Fig. 2.5a). Both cladograms indicate significant morphometric differentiation between *Z. senegalensis* and the rest of the southern African taxa (BS = 100, Fig. 2.5a). In addition the discrete character analysis (Fig. 2.5a ii), but not the continuous character analysis (Fig. 2.5ai), indicates that *Z. p. haigamchabensis* also differs from the rest of the South African *Zosterops* taxa, although support for this is low (BS = 52).

The univariate analyses of the quantitative colour score data (Table 2.7, Fig. 2.6 – 2.9) provided 11 qualitative characters (either bi- or multi-state) for use in the parsimony analyses. Only the quantitative score for the character "right flank green" produced no variation among taxa. Single most parsimonious trees were obtained for both the continuous and discrete character data sets (Fig. 2.5b). The topologies between the two data sets differ, with the continuous character parsimony tree supporting only the separation of *Z. senegalensis* from the rest of the southern African *Zosterops* taxa (BS = 100; Fig. 2.5bi). Although the bootstrap value was relatively low (BS = 54), the discrete tree (Fig. 2.5bii) groups the grey-bellied birds (*Z. v. atmorii* and *Z. v. capensis*) with the Orange River white-eye taxa (*Z. p. sundevalli, Z. p. deserticola* and *Z. p. haigamchabensis*). *Zosterops vaalensis* is sister to the grey-bellied and Orange River clade, but with weak support (BS = 44), effectively forming a polytomy.

The uni-variate analyses of the percentage colour (Table 2.8, Fig. 2.10) data set also provided qualitative characters (either bi- or multi-state) for use in the parsimony analyses. A single most parsimonious tree was recovered for the continuous data (Fig. 2.5ci) and, three equally parsimonious trees were recovered for the discrete data, from which a strict consensus tree was produced (Fig. 2.5cii). Both trees have similar topologies of four clades; however, the discrete tree (Fig. 2.5cii) resulted in greater taxonomic resolution. The grey-

bellied birds and Orange River birds (including *Z. vaalensis*) are sister groups in the discrete tree (BS = 67).

The combined data set produced a single most parsimonious tree for the continuous data (Fig. 2.5di) and three equally parsimonious trees were recovered for the discrete data, from which a strict consensus tree was produced (Fig. 2.5dii). Although bootstrap values were slightly lower, similar topologies as for the continuous and discrete (Fig. 2.5c) parsimony trees of the percentage colour data set were obtained, with grey-bellied birds and Orange River birds (including *Z. vaalensis*) as sister groups.

2.5. Discussion

In order for the subspecies concept to be useful in systematics, as opposed to being representative of transient eco-geographical variation, taxa described as subspecies must exhibit their own evolutionary trajectory and not be clinal variants based on a single character (Mayr and Ashlock 1991, Barrowclough 1982, Patten and Unitt 2002, Zink 2004, Rising 2007, Fitzpatrick 2010) but should be defined or described through unique combinations of character states. Discontent with subspecies as a concept (Wilson and Brown 1953, Zink 2004) apparently often results from the inappropriate use in the past (Patten and Unitt 2002, Remsen 2010, Winker 2010), with several authors suggesting (Rising 2007, Remsen 2010, Winker 2010) the need for the use of a least one diagnosable morphometric trait, for the justifiable use of the subspecies.

Classifications of southern African *Zosterops* taxa (Gill 1936, Roberts 1942, Moreau 1957, Clancey 1967, Skead 1967, Hockey *et al*. 2005, van Balen 2008) have varied considerably in the number of taxa described (Table 2.1), with underpart plumage variation being the primary line of evidence in delimiting the number of *Zosterops* taxa. However, no consensus has yet been reached. This chapter has attempted to objectively establish the

number of southern African *Zosterops* ESU using those characters available to past taxonomists by employing modern uni- and multi-variate statistical techniques. These newly delineated taxonomic units will form the basis for further evaluation in the following chapters.

2.5.1. *Putative* Zosterops *taxa*

Four data sets were analysed using uni- and multi-variate statistical approaches to determine whether the nine southern African *Zosterops* taxa described by previous taxonomists (Table 2.1) can be accepted as true ESU. On their own, the morphometric data was only able to distinguish between *Z. senegalensis* and the southern African *Zosterops* taxa (except *Z. p. haigamchabensis*; Table 2.2). Parsimony (Fig. 2.5) and MDS analyses (Fig. 2.3) of the morphometric data also indicate a lack of taxonomic partitioning among southern African *Zosterops* taxa.

Underpart plumage colouration of African *Zosterops* taxa is viewed as the primary means by which to distinguish among taxa (Gill 1936, Roberts 1942, Moreau 1957, Clancy 1967, Skead 1967). The results here support this view. Both colour assessment data sets group birds according to underpart colouration (Fig. 2.3 & 2.5). However, the grouping of the MDS analyses does differ between the quantitative colour scores and the percentage colour data scores. Although the quantitative colour scores are very broad in its grouping, green-bellied *Z. v. virens* and *Z. v. caniviridis* individuals, as well as some *Z. vaalensis* individuals do group together (Fig. 2.3b). The Orange River and grey-bellied birds are not well separated (Fig. 2.3b). The tighter groupings from the percentage colour data (Fig. 2.3c) strongly suggest the presence of four *Zosterops* taxa in southern Africa (see below). The placement of *Z. vaalensis* individuals within both the green-bellied and Orange River group (Fig. 2.3c), and not as a grouping on their own, indicate that these birds may be merely intermediates and not

separate entities as suggested in the past (Gill 1936, Roberts 1942). The African Yellow White-eye, *Z. senegalensis*, is found mixed with green South African birds, as well as forming a separate group in the percentage colour MDS analysis (Fig. 2.3c). Individuals of *Z. senegalensis* found to group with the green-bellied birds (Fig. 2.4) are described as subspecies (e.g. *Z. s. stenocrita*, *Z. s. stuhlmanni*, *Z. s. toruensis*, *Z. s. kasiacus* and *Z. s. reichenowi*) that tend to be much greener underneath than the nominate *Z. s. senegalensis* (Fry *et al*. 2000, van Balen 2008). Those subspecies described as similar in colouration to *Z. s. senegalensis* group together (Fig. 2.3c) and include: *Z. s. demeryi*, *Z. s. jacksoni*, *Z. s. stierlingi* and *Z. s. anderssoni*.

2.5.2. *Four* Zosterops *taxa*

The combined percentage colour and morphometric analysis support the recognition of four southern African *Zosterops* ESU: the African Yellow White-eye *Zosterops senegalensis*; the Green White-eye *Z. virens* (*Z. v. virens* and *Z. v. caniviridis*); the Cape White-eye *Z. capensis* (*Z. v. atmorii* and *Z. v. capensis*) and, the Orange River White-eye *Z. pallidus* (*Z. pallidus sundevalli*, *Z. p. deserticola* and *Z. p. haigamchabensis*). *Z. vaalensis* is considered to be a hybrid between *Z. virens* and *Z. pallidus*.

A discriminant function analysis of the four putative *Zosterops* taxa performed on the morphometric measurements diagnosed 68.3% (Table 2.9) of the birds correctly. However, only *Z. senegalensis* and *Z. virens* were readily diagnosable, 86.9% and 89.7% respectively. Thus, the discriminatory ability of the morphometric data is weak for these four taxa and hence it would be unwise to base *Zosterops* taxonomic conclusions solely on these measurements. The addition of the percentage colour scores to the morphometric discriminant function analysis increased our ability to diagnose the four taxa to 98.4% (Table 2.10); all four taxa could be readily diagnosed (> 90%). *Zosterops capensis* was recovered

100% correctly. Incorrectly observed birds of *Z. senegalensis* were identified as *Z. virens*, and vice versa. *Zosterops pallidus* individuals that were incorrectly classified were identified as *Z. virens* (3 individuals) and *Z. capensis* (1 individual). The three individuals identified as *Z. virens* possess intermediate underpart colouration and were collected in the area of overlap between *Z. pallidus* and *Z. virens* and therefore may represent hybrids between these two taxa. Given the high diagnosability of taxa from the discriminant functions analyses, species status is recognized for the four *Zosterops* taxa.

Crandall *et al.* (2000) argue that conservation effort should be placed on subspecies or populations that possess high functional diversity, thereby increasing the potential for species survival, although others disagree (Moritz 1994, Zink 2004). Geographically isolated populations within a species may exhibit relatively large amounts of potentially adaptive variation (Crandall *et al.* 2000) and can provide insight to the processes that drive evolution and speciation (Rising 2007). Zink (2004), in contrast, argues that only demonstrably reciprocally monophyletic populations that may have taken thousands of years to evolve warrant ESU status. Thus, under current avian taxonomy, most named subspecies in the Palearctic and Nearctic should be considered to be simply geographical, clinally varying populations, often without any apparent distinct evolutionary significance (Zink 2004). In contrast, Phillimore and Owens (2006) suggest that many island and other continental regions (Afrotropics, Indo-Malaysia and Neotropics) exhibit greater concordance (36%) between subspecies delineation and the recognition of distinct lineages.

Although on the increase, molecular phylogenetic studies of Africa avian taxa are still relatively sparse. Biogeographic areas with little molecular evidence (Beheregaray 2009) have to rely on traditional taxonomic units, whether they are at the species or subspecies level, when deciding on conservation priorities. Rigorous reassessments of morphological subspecific classifications can determine whether they correctly identify historically

meaningful entities or if they only recognize subtle variations across changes in eco-geographical space, both of which are interesting evolutionary phenomena.

A thorough examination of all available morphological (morphometrics and plumage colouration) evidence has resulted in the recognition of four discernable southern African *Zosterops* species. They are: Grey Cape White-eye *Z. capensis*, Orange River White-eye *Z. pallidus*, African Yellow White-eye *Z. senegalensis* and Green Cape White-eye *Z. virens*. The remaining chapters will test the validity of these proposed morphologically defined species by incorporating both vocal (Chapter 3) and mitochondrial and nuclear DNA (Chapter 4 and 5).

Tables

Table 2.1: Taxonomic treatment of the grey-bellied and green-bellied Cape White-eyes, Orange River White-eye and African Yellow White-eye.

Author	Grey Cape White-eye	Green Cape White-eye	Orange River White-eye	African Yellow White-eye	Additional taxa
Gill (1936)	*Z. capensis*	*Z. virens*	*Z. pallidus*	*Z. flaviventris*	*Z. vaalensis*
Roberts (1942)	*Z. capensis* (5 subspecies: *Z. c. capensis, Z. c. atmorii, Z. c. basuticus, Z. c. pallidus, Z. c. deserticola)*	*Z. virens*	*Z. c. pallidus*	*Z. senegalensis*	*Z. vaalensis*
Moreau (1957)	*Z. v. capensis* & *Z. v. atmorii*	*Z. v. virens*	*Z. pallidus*	*Z. andersonii*	
Skead (1967)	*Z. v. capensis* & *Z. v. atmorii*	*Z. v. virens* & *Z. v. caniviridis*	*Z. p. pallidus* & *Z. p. sundevalli*	*Z. senegalensis*	
Clancey (1967)	*Z. p. capensis*	*Z. p. virens*	*Z. p. pallidus*	*Z. senegalensis*	
Hockey *et al*. (2005)	*Z. v. capensis* (incl. *Z. v. atmorii*)	*Z. v. virens* (incl. *Z. v. caniviridis*)	*Z. p. sundevalli, Z. p. deserticola* & *Z. p. haigamchabensis*	*Z. s. andersoni*	
van Balen (2008)	*Z. p. capensis* & *Z. p. atmorii*	*Z. p. virens* & *Z. p. caniviridis*	*Z. p. pallidus* & *Z. p. sundevalli*	*Z. s. andersoni*	

Table 2.2: The mean and standard error (SE) of the morphometric assessments for the nine southern African *Zosterops* taxa. Significant differences between species were tested using one-way ANCOVA with a post-hoc Newman-Keuls test to determine which taxa differed.

Character	mean ± SE (mm)	
Wing-length (mm)		$F = 37.26$, df = 556, $P < 0.001$
Z. senegalensis (n = 46)	56.40 ± 0.50	can, vir, atm, cap, vaal, sun, des > sen = haig
Z. v. caniviridis (n = 39)	61.29 ± 0.24	can > vir, atm, cap, vaal, can, des > sen, haig
Z. v. virens (n = 233)	60.02 ± 0.16	can > atm, cap, vaal, sun, des = vir > sen, haig
Z. v. atmorii (n = 51)	59.85 ± 0.33	can > vir, cap, vaal, sun, des = atm > sen, haig
Z. v. capensis (n = 51)	60.06 ± 0.41	can > vir, atm, vaal, sun, des = cap > sen, haig
Z. vaalensis (n = 27)	59.80 ± 0.58	can > vir, cap, atm, sun, des = vaal > sen, haig
Z. p. sundevalli (n = 40)	58.60 ± 0.31	can > vir, atm, cap, vaal, des = sun > sen, haig
Z. p. deserticola (n = 60)	57.63 ± 0.20	can > vir, atm, cap, vaal, sun = des > sen, haig
Z. p. haigamchabensis (n = 10)	55.70 ± 0.52	sen = haig < can, vir, atm, cap, vaal, sun, des
Bill-length (mm)		$F = 68.92$, df = 556, $P < 0.001$
Z. senegalensis (n = 46)	12.66 ± 0.14	sen > can, vir, atm, cap, vaal, sun, des, haig
Z. v. caniviridis (n = 39)	10.15 ± 0.10	sen > can = vir, atm, cap > vaal, sun, des, haig
Z. v. virens (n = 233)	10.34 ± 0.04	sen > vir = can, atm, cap > vaal, sun, des, haig
Z. v. atmorii (n = 51)	10.52 ± 0.09	sen > atm = can, vir, cap > vaal, sun, des, haig
Z. v. capensis (n = 51)	10.35 ± 0.10	sen > cap = can, vir, atm > vaal, sun, des, haig
Z. vaalensis (n = 27)	9.67 ± 0.22	sen > can, vir, atm, cap > vaal = sun, des, haig

Z. p. sundevalli (n = 40)	9.65 ± 0.11	sen > can, vir, atm, cap > sun = vaal, des, haig
Z. p. deserticola (n = 60)	9.73 ± 0.08	sen > can, vir, atm, cap > des = vaal, sun, haig
Z. p. haigamchabensis (n = 10)	9.10 ± 0.08	sen > can, vir, atm, cap > haig = vaal, sun, des
Tail-length (mm)	$F = 12.74$, df = 556, $P < 0.001$	
Z. senegalensis (n = 46)	44.03 ± 0.75	can, vir, atm, cap, vaal, sun, des > sen = haig
Z. v. caniviridis (n = 39)	45.95 ± 0.46	sen, haig < can = vir, atm, cap, vaal, sun, des
Z. v. virens (n = 233)	45.73 ± 0.18	sen, haig < vir = can, atm, cap, vaal, sun, des
Z. v. atmorii (n = 51)	47.76 ± 0.48	sen, haig < atm = can, vir, cap, vaal, sun, des
Z. v. capensis (n = 51)	47.90 ± 0.44	sen, haig < cap = can, vir, atm, vaal, sun, des
Z. vaalensis (n = 27)	47.47 ± 0.77	sen, haig < vaal = can, vir, atm, cap, sun, des
Z. p. sundevalli (n = 40)	46.93 ± 0.34	sen, haig < sun = can, vir, atm, cap, vaal, des
Z. p. deserticola (n = 60)	46.21 ± 0.27	sen, haig < des = can, vir, atm, cap, vaal, sun
Z. p. haigamchabensis (n = 10)	43.85 ± 0.55	can, vir, atm, cap, vaal, sun, des > haig = sen

Table 2.3: A Discriminant Function Analysis matrix indicating the observed classification percentages using the morphometric data for the nine southern African *Zosterops* taxa. sen = *Z. senegalensis*, can = *Z. virens caniviridis*, vir = *Z. v. virens*, atm = *Z. v. atmorii*, cap = *Z. v. capensis*, vaal = *Z. vaalensis*, sun = *Z. pallidus sundevalli*, des = *Z. pallidus deserticola*, haig = *Z. pallidus haigamchabensis*.

Taxa	**% Correct**	**sen**	**can**	**vir**	**atm**	**cap**	**vaal**	**sun**	**des**	**haig**
sen	91.30	42	0	4	0	0	0	0	0	0
can	0.00	0	0	39	0	0	0	0	1	0
vir	93.54	3	1	217	1	0	0	1	8	1
atm	3.85	0	0	43	2	0	0	0	7	0
cap	0.00	1	1	37	2	0	0	5	4	1
vaal	0.00	0	0	9	0	0	0	3	1	1
sun	21.74	0	1	24	0	0	0	10	8	3
des	16.42	0	0	49	1	0	0	6	11	0
haig	10.00	0	0	2	0	0	0	6	0	1
Total	**50.72**	**46**	**3**	**425**	**6**	**0**	**0**	**31**	**40**	**7**

Table 2.4: A Discriminant Function Analysis matrix indicating the observed classification percentages using the quantitative colour data for the nine southern African *Zosterops* taxa. sen = *Z. senegalensis*, can = *Z. virens caniviridis*, vir = *Z. v. virens*, atm = *Z. v. atmorii*, cap = *Z. v. capensis*, vaal = *Z. vaalensis*, sun = *Z. pallidus sundevalli*, des = *Z. pallidus deserticola*, haig = *Z. pallidus haigamchabensis*.

Taxa	% Correct	sen	can	vir	atm	cap	vaal	sun	des	haig
sen	100.00	3	0	0	0	0	0	0	0	0
can	22.50	0	9	31	0	0	0	0	0	0
vir	93.97	0	6	218	4	3	1	0	0	0
atm	84.62	0	0	0	44	8	0	0	0	0
cap	76.47	0	0	0	12	39	0	0	0	0
vaal	80.00	0	0	1	0	1	12	1	0	0
sun	51.11	0	0	0	2	1	0	23	19	0
des	76.12	0	0	0	0	1	1	14	51	0
haig	90.00	0	0	0	0	1	0	0	0	9
Total	**79.22**	**3**	**15**	**250**	**62**	**54**	**14**	**38**	**70**	**9**

Table 2.5: A Discriminant Function Analysis matrix indicating the observed classification percentages using the percentage colour data for the nine southern African *Zosterops* taxa. sen = *Z. senegalensis*, can = *Z. virens caniviridis*, vir = *Z. v. virens*, atm = *Z. v. atmorii*, cap = *Z. v. capensis*, vaal = *Z. vaalensis*, sun = *Z. pallidus sundevalli*, des = *Z. pallidus deserticola*, haig = *Z. pallidus haigamchabensis*.

Taxa	% Correct	sen	can	vir	atm	cap	vaal	sun	des	haig
sen	41.30	19	0	27	0	0	0	0	0	0
can	0.00	3	0	37	0	0	0	0	0	0
vir	99.14	2	0	230	0	0	0	0	0	0
atm	48.08	0	0	0	25	27	0	0	0	0
cap	84.31	0	0	0	8	43	0	0	0	0
vaal	33.33	0	0	3	0	0	5	1	4	2
sun	31.11	0	0	0	1	0	1	14	24	5
des	82.09	0	0	0	0	0	0	10	55	2
haig	30.00	0	0	0	1	0	1	4	1	3
Total	**70.61**	**24**	**0**	**297**	**35**	**70**	**7**	**29**	**84**	**12**

Table 2.6: A Discriminant Function Analysis matrix indicating the observed classification percentages using the combined data for the nine southern African *Zosterops* taxa. sen = *Z. senegalensis*, can = *Z. virens caniviridis*, vir = *Z. v. virens*, atm = *Z. v. atmorii*, cap = *Z. v. capensis*, vaal = *Z. vaalensis*, sun = *Z. pallidus sundevalli*, des = *Z. pallidus deserticola*, haig = *Z. pallidus haigamchabensis*.

Taxa	% Correct	sen	can	vir	atm	cap	vaal	sun	des	haig
sen	89.13	41	1	4	0	0	0	0	0	0
can	2.50	0	1	39	0	0	0	0	0	0
vir	99.57	1	0	231	0	0	0	0	0	0
atm	50.00	0	0	0	26	26	0	0	0	0
cap	80.39	0	0	0	10	41	0	0	0	0
vaal	42.86	0	0	2	0	0	6	2	3	1
sun	36.96	0	0	0	0	0	1	17	24	4
des	79.10	0	0	0	0	0	0	12	53	2
haig	40.00	0	0	0	1	0	0	4	1	4
Total	**75.27**	**42**	**2**	**276**	**37**	**67**	**7**	**35**	**81**	**11**

Table 2.7: The mean and standard error (SE) of the quantitative colour scores for the nine southern African *Zosterops* taxa. Significant differences between species were tested using one-way ANOVA with a post-hoc Newman-Keuls test to determine which taxa differed.

Character	mean ± SE	
Throat red		$F = 13.31$, df = 513, $P < 0.001$
Z. senegalensis (n= 3)	189.44 ± 9.86	*
Z. v. caniviridis (n = 39)	195.66 ± 9.77	sen, vir, atm, vaal, sun, des, haig = can > cap
Z. v. virens (n = 233)	187.79 ± 11.14	*
Z. v. atmorii (n = 51)	186.08 ± 10.08	*
Z. v. capensis (n = 51)	174.99 ± 11.74	can, des > cap = sen, vir, atm, vaal, sun, haig
Z. vaalensis (n = 27)	187.40 ± 9.07	*
Z. p. sundevalli (n = 40)	188.64 ± 10.58	*
Z. p. deserticola (n = 60)	192.37 ± 9.99	sen, can, vir, atm, vaal, sun, haig = des > cap
Z. p. haigamchabensis (n = 10)	191.93 ± 16.65	*
Throat green		$F = 14.62$, df = 513, $P < 0.001$
Z. senegalensis (n = 3)	180.22 ± 9.02	*
Z. v. caniviridis (n = 39)	190.40 ± 9.86	sen, vir, atm, vaal, sun, des, haig = can > cap
Z. v. virens (n – 233)	181.60 ± 11.49	*
Z. v. atmorii (n = 51)	179.22 ± 10.72	*
Z. v. capensis (n = 51)	168.31 ± 10.41	can, des > cap = sen, vir, atm, vaal, sun, haig
Z. vaalensis (n = 27)	181.11 ± 10.08	*
Z. p. sundevalli (n = 40)	183.24 ± 10.15	*
Z. p. deserticola (n = 60)	185.94 ± 9.93	can, des > cap = sen, vir, atm, vaal, sun, haig
Z. p. haigamchabensis (n = 10)	176.13 ± 16.80	*

Throat blue		$F = 32.06$, df = 513, $P < 0.001$
Z. senegalensis (n = 3)	52.33 ± 14.19	vaal, sun, des, haig > sen = can, vir, atm, cap
Z. v. caniviridis (n = 39)	69.94 ± 13.24	sun, des > can = sen, vir, atm, cap, vaal, haig
Z. v. virens (n = 233)	65.94 ± 17.39	sun, des > vir = sen, can, atm, cap, vaal, haig
Z. v. atmorii (n = 51)	73.92 ± 18.03	sun > atm = sen, can, vir, cap, vaal, des, haig
Z. v. capensis (n = 51)	71.72 ± 16.01	sun > cap = sen, can, vir, atm, vaal, des, haig
Z. vaalensis (n = 27)	84.69 ± 23.17	can, vir, atm, cap, sun, des, haig = vaal > sen
Z. p. sundevalli (n = 40)	98.84 ± 23.22	vaal, des, haig = sun > sen, can, vir, atm, cap
Z. p. deserticola (n = 60)	95.19 ± 14.28	atm, cap, vaal, sun, haig = des > sen, can, vir,
Z. p. haigamchabensis (n = 10)	87.33 ± 17.62	can, vir, atm, cap, vaal, sun, des = haig > sen
Belly red		$F = 16.34$, df = 513, $P < 0.001$
Z. senegalensis (n = 3)	201.44 ± 7.63	*
Z. v. caniviridis (n = 39)	202.95 ± 11.24	sen, vir, atm, vaal, sun, des, haig = can > cap
Z. v. virens (n = 233)	194.33 ± 13.05	*
Z. v. atmorii (n = 51)	192.31 ± 13.85	*
Z. v. capensis (n = 51)	181.35 ± 13.76	can, vaal, sen, des > cap = sen, vir, atm, haig
Z. vaalensis (n = 27)	198.29 ± 8.42	sen, can, vir, atm, sun, des, haig = vaal > cap
Z. p. sundevalli (n = 40)	199.77 ± 10.88	sen, can, vir, atm, vaal, des, haig = sun> cap
Z. p. deserticola (n = 60)	204.35 ± 9.50	sen, can, vir, atm, vaal, sun = des> cap, haig
Z. p. haigamchabensis (n = 10)	187.03 ± 17.50	des > haig = sen, can, vir, atm, cap, vaal, sun
Belly green		$F = 16.35$, df = 513, $P < 0.001$
Z. senegalensis (n = 3)	188.00 ± 9.02	*
Z. v. caniviridis (n = 39)	194.61 ± 11.66	sen, vir, atm, vaal, sun, des, haig = can > cap
Z. v. virens (n = 233)	184.74 ± 13.20	*
Z. v. atmorii (n = 51)	185.29 ± 14.48	*
Z. v. capensis (n = 51)	174.52 ± 13.63	sen, can, vaal, sen, des > cap = vir, atm, haig
Z. vaalensis (n =27)	187.71 ± 9.73	sen, can, vir, atm, sun, des, haig = vaal > cap
Z. p. sundevalli (n = 40)	193.30 ± 11.55	sen, can, vir, atm, vaal, des, haig = sun> cap
Z. p. deserticola (n = 60)	196.87 ± 9.52	sen, can, vir, atm, vaal, sun = des> cap, haig
Z. p. haigamchabensis (n = 10)	176.57 ± 17.95	des > haig = sen, can, vir, atm, cap, vaal, sun
Belly blue		$F = 333.91$, df = 513, $P < 0.001$
Z. senegalensis (n = 3)	43.56 ± 8.00	sen < can, vir, atm, cap, vaal, sun, des, haig
Z. v. caniviridis (n = 39)	93.45 ± 15.45	atm, cap, vaal, sun, des, haig > can = vir > sen
Z. v. virens (n = 233)	89.15 ± 18.29	atm, cap, vaal, sun, des, haig > vir = can > sen
Z. v. atmorii (n =51)	166.11 ± 16.15	cap, sun, des, haig = atm > sen, can, vir, vaal
Z. v. capensis (n = 51)	155.12 ± 12.76	atm, vaal, sun, des, haig = cap > sen, can, vir
Z. vaalensis (n = 27)	117.53 ± 26.68	atm, sun, des > vaal = cap, haig > sen, can, vir
Z. p. sundevalli (n = 40)	170.94 ± 15.56	atm, cap, des, haig = sun > sen, can, vir, vaal
Z. p. deserticola (n = 60)	171.74 ± 13.70	atm, cap, sun, haig = des > sen, can, vir, vaal
Z. p. haigamchabensis (n = 10)	150.23 ± 19.92	atm, cap, vaal, sun, des = haig > sen, can, vir
Right flank red		$F = 31.69$, df = 513, $P < 0.001$
Z. senegalensis (n = 3)	171.00 ± 7.54	can, vaal, sun, des, haig = sen > vir, atm, cap
Z. v. caniviridis (n = 39)	160.26 ± 13.12	sen, vir, cap, vaal, sun, des, haig = can > atm
Z. v. virens (n = 233)	150.40 ± 11.22	sen, vaal, des, haig > vir = can, atm, cap, sun
Z. v. atmorii (n = 51)	146.87 ± 12.65	sen, vaal, sun, des, haig > vir = can, atm, cap

Z. v. capensis (n = 51)	148.39 ± 13.81	sen, vaal, des, haig > cap = can, vir, atm, sun
Z. vaalensis (n = 27)	160.33 ± 6.50	sen, can, sun, des, haig = vaal > vir, atm, cap
Z. p. sundevalli (n = 40)	163.13 ± 11.91	sen, can, vir, cap, vaal, des, haig = sun > atm
Z. p. deserticola (n = 60)	169.48 ± 9.71	sen, can, vaal, sun, haig = des > vir, atm, cap
Z. p. haigamchabensis (n = 10)	174.30 ± 7.90	sen, can, vaal, sun, des = haig > vir, atm, cap

Right flank green		$F = 6.56$, df = 513, $P < 0.001$
Z. senegalensis (n = 3)	162.33 ± 6.93	can, vaal, des, haig = sen > vir, atm, cap, sun
Z. v. caniviridis (n = 39)	153.29 ± 12.07	*
Z. v. virens (n = 233)	143.46 ± 10.80	sen > vir = can, atm, cap, vaal, sun, des, haig
Z. v. atmorii (n = 51)	139.91 ± 12.38	sen > atm = can, vir, cap, vaal, sun, des, haig
Z. v. capensis (n = 51)	142.10 ± 14.67	sen > cap = can, vir, atm, vaal, sun, des, haig
Z. vaalensis (n = 27)	143.31 ± 9.02	*
Z. p. sundevalli (n = 40)	144.41 ± 13.92	sen > sun = can, vir, atm, cap, vaal, des, haig
Z. p. deserticola (n = 60)	148.38 ± 10.82	*
Z. p. haigamchabensis (n = 10)	151.40 ± 10.68	*

Right flank blue		$F = 108.85$, df = 513, $P < 0.001$
Z. senegalensis (n = 3)	56.22 ± 5.17	sen < can, vir, atm, cap, vaal, sun, des, haig
Z. v. caniviridis (n = 39)	82.10 ± 14.91	atm, cap, vaal, sun, des, haig > can = vir > sen
Z. v. virens (n = 233)	77.53 ± 15.53	atm, cap, vaal, sun, des, haig > vir = can > sen
Z. v. atmorii (n = 51)	125.68 ± 14.13	cap, vaal, sun, des, haig = atm > sen, can, vir
Z. v. capensis (n = 51)	127.05 ± 16.58	atm, sun, des, haig = cap > sen, can, vir, vaal
Z. vaalensis (n = 27)	89.13 ± 18.70	cap > vaal = atm, sun, des, haig > sen, can, vir
Z. p. sundevalli (n = 40)	110.55 ± 19.25	atm, cap, vaal, des, haig = sun > sen, can, vir
Z. p. deserticola (n = 60)	111.80 ± 15.26	atm, cap, vaal, sun, haig = des > sen, can, vir
Z. p. haigamchabensis (n = 10)	112.40 ± 22.24	atm, cap, vaal, sun, des = haig > sen, can, vir

Left flank red		$F = 24.94$, df = 513, $P < 0.001$
Z. senegalensis (n = 3)	163.89 ± 5.01	sen = can, vir, vaal, sun, des, haig > atm, cap
Z. v. caniviridis (n = 39)	160.52 ± 10.77	*
Z. v. virens (n = 233)	150.59 ± 11.47	*
Z. v. atmorii (n = 51)	145.05 ± 11.43	sen, des > atm = can, vir, cap, vaal, sun, haig
Z. v. capensis (n = 51)	144.86 ± 13.20	sen, des > cap = can, vir, atm, vaal, sun, haig
Z. vaalensis (n = 27)	157.84 ± 10.04	*
Z. p. sundevalli (n = 40)	158.56 ± 8.84	*
Z. p. deserticola (n = 60)	166.19 ± 9.11	sen, can, vir, vaal, sun, haig = des > atm, cap
Z. p. haigamchabensis (n = 10)	156.37 ± 8.44	*

Left flank green		$F = 10.16$, df = 513, $P < 0.001$
Z. senegalensis (n = 3)	151.11 ± 5.21	*
Z. v. caniviridis (n = 39)	154.09 ± 10.04	sen, vir, atm, vaal, sun, des, haig = can > cap
Z. v. virens (n = 233)	143.72 ± 11.28	*
Z. v. atmorii (n = 51)	137.78 ± 12.09	*
Z. v. capensis (n = 51)	137.22 ± 13.60	can > cap = sen, vir, atm, vaal, sun, des, haig
Z. vaalensis (n = 27)	140.29 ± 11.74	*
Z. p. sundevalli (n = 40)	138.69 ± 9.06	*
Z. p. deserticola (n = 60)	145.70 ± 9.09	*
Z. p. haigamchabensis (n = 10)	139.63 ± 8.22	*

Left flank blue	$F = 110.68$, df = 513, $P < 0.001$	
Z. senegalensis (n = 3)	43.00 ± 7.86	sen < can, vir, atm, cap, vaal, sun, des, haig
Z. v. caniviridis (n = 39)	82.04 ± 12.00	atm, cap, sun, des, haig > can = vir, vaal > sen
Z. v. virens (n = 233)	77.29 ± 15.42	atm, cap, sun, des, haig > vir = can, vaal > sen
Z. v. atmorii (n = 51)	123.10 ± 14.83	cap, sun, des, haig = atm > sen, can, vir, vaal
Z. v. capensis (n = 51)	121.29 ± 14.80	atm, sun, des, haig = cap > sen, can, vir, vaal
Z. vaalensis (n = 27)	87.51 ± 21.52	atm, cap > vaal = can, vir, sun, des, haig > sen
Z. p. sundevalli (n = 40)	104.13 ± 13.48	atm, cap, vaal, des, haig =sun > sen, can, vir
Z. p. deserticola (n = 60)	110.77 ± 12.23	atm, cap, vaal, sun, haig =des > sen, can, vir
Z. p. haigamchabensis (n = 10)	104.33 ± 16.30	atm, cap, vaal, sun, des =haig > sen, can, vir

* no significant difference among taxa

Table 2.8: The mean and standard error (SE) of the percentage colour assessments for the nine southern African *Zosterops* taxa. Significant differences between species were tested using one-way ANOVA with a post-hoc Newman-Keuls test to determine which taxa differed.

Character	mean ± SE	
Percentage grey	$F = 1488.8$, df = 556, $P < 0.001$	
Z. senegalensis (n = 46)	0.00 ± 0.00	atm, cap, haig > sen = can, vir, vaal, sun, des
Z. v. caniviridis (n = 39)	0.00 ± 0.00	atm, cap, haig > can = sen, vir, vaal, sun, des
Z. v. virens (n = 233)	0.00 ± 0.00	atm, cap, haig > vir = sen, can, vaal, sun, des
Z. v. atmorii (n = 51)	60.00 ± 11.09	cap = atm < sen, can, vir, vaal, sun, des, haig
Z. v. capensis (n = 51)	64.00 ± 8.99	atm = cap < sen, can, vir, vaal, sun, des, haig
Z. vaalensis (n = 27)	1.00 ± 2.40	atm, cap, haig > vaal = sen, can, vir, sun, des
Z. p. sundevalli (n = 40)	3.00 ± 8.11	atm, cap > sun = sen, can, vir, vaal, des, haig
Z. p. deserticola (n = 60)	0.00 ± 2.63	atm, cap, haig > des = sen, can, vir, vaal, sun
Z. p. haigamchabensis (n = 10)	7.00 ± 20.38	atm, cap > haig = sun < sen, can, vir, vaal, des
Percentage green	$F = 788.02$, df = 556, $P < 0.001$	
Z. senegalensis (n = 46)	49.00 ± 25.60	can, vir > sen < atm, cap, vaal, sun, des, haig
Z. v. caniviridis (n = 39)	65.00 ± 8.71	vir = can > sen, atm, cap, vaal, sun, des, haig
Z. v. virens (n = 233)	71.00 ± 7.55	can = vir > sen, atm, cap, vaal, sun, des, haig
Z. v. atmorii (n = 51)	0.00 ± 0.00	sen, can, vir, vaal > atm = cap, sun, des, haig
Z. v. capensis (n = 51)	0.00 ± 0.00	sen, can, vir, vaal > cap = atm, sun, des, haig
Z. vaalensis (n = 27)	23.00 ± 23.73	sen, can, vir > vaal < atm, cap, sun, des, haig
Z. p. sundevalli (n = 40)	2.00 ± 11.47	sen, can, vir, vaal > sun = atm, vir, des, haig
Z. p. deserticola (n = 60)	0.00 ± 0.00	sen, can, vir, vaal > des = atm, vir, sun, haig
Z. p. haigamchabensis (n = 10)	0.00 ± 0.00	sen, can, vir, vaal > haig = atm, vir, sun, des
Percentage cinnamon	$F = 761.93$, df = 556, $P < 0.001$	
Z. senegalensis (n = 46)	0.00 ± 0.00	vaal, sun, des, haig > sen = can, vir, atm, cap
Z. v. caniviridis (n = 39)	0.00 ± 0.00	vaal, sun, des, haig > can = sen, vir, atm, cap
Z. v. virens (n = 233)	0.00 ± 0.00	vaal, sun, des, haig > vir = sen, can, atm, cap
Z. v. atmorii (n = 51)	3.00 ± 8.94	vaal, sun, des, haig > atm = sen, can, vir, cap
Z. v. capensis (n = 51)	2.00 ± 5.89	vaal, sun, des, haig > cap = sen, can, vir, atm

Z. vaalensis (n = 27)	36.00 ± 12.34	sun, des > vaal = haig > sen, can, vir, atm, cap
Z. p. sundevalli (n = 40)	42.00 ± 12.01	sun = des, haig > sen, can, vir, atm, cap, vaal
Z. p. deserticola (n = 60)	44.00 ± 7.68	des = sun, haig > sen, can, vir, atm, cap, vaal
Z. p. haigamchabensis (n = 10)	40.00 ± 13.26	haig = sun, des > sen, can, vir, atm, cap, vaal
Percentage yellow	$F = 24.74$, df = 556, $P < 0.001$	
Z. senegalensis (n = 46)	51.00 ± 25.60	sun = haig > can, vir, atm, cap, vaal, sun, des
Z. v. caniviridis (n = 39)	35.00 ± 8.64	sun > can = vir, atm, cap, vaal, sun, des, haig
Z. v. virens (n = 233)	30.00 ± 7.56	sun, haig > vir = can, atm, cap, vaal, sun, des
Z. v. atmorii (n = 51)	33.00 ± 7.08	sun, haig > atm = can, vir, cap, vaal, sun, des
Z. v. capensis (n = 51)	34.00 ± 7.35	sun, haig > cap = can, vir, atm, vaal, sun, des
Z. vaalensis (n = 27)	35.00 ± 9.71	sun, haig > vaal = can, vir, atm, cap, sun, des
Z. p. sundevalli (n = 40)	37.00 ± 5.07	sun > sun = can, vir, atm, cap, vaal, des, haig
Z. p. deserticola (n = 60)	37.00 ± 6.23	sun > des = can, vir, atm, cap, vaal, sun, haig
Z. p. haigamchabensis (n = 10)	45.00 ± 11.19	haig = sen, can, sun, des > vir, atm, cap, vaal
Percentage white	$F = 142.13$, df = 556, $P < 0.001$	
Z. senegalensis (n = 46)	0.00 ± 0.00	sen = can, vir, atm, cap, vaal < sun, des, haig
Z. v. caniviridis (n = 39)	0.00 ± 0.00	can = sen, vir, atm, cap, vaal < sun, des, haig
Z. v. virens (n = 233)	0.00 ± 0.00	vir = sen, can, atm, cap, vaal < sun, des, haig
Z. v. atmorii (n = 51)	4.00 ± 5.66	atm = sen, can, vir, cap, vaal < sun, des, haig
Z. v. capensis (n = 51)	1.00 ± 2.54	cap = sen, can, vir, atm, vaal < sun, des, haig
Z. vaalensis (n = 27)	6.00 ± 9.43	vaal = sen, can, vir, atm, cap, des < sun, haig
Z. p. sundevalli (n = 40)	16.00 ± 9.81	sun = des > sen, can, vir, atm, cap, vaal, haig
Z. p. deserticola (n = 60)	20.00 ± 9.96	des = sun > sen, can, vir, atm, cap, vaal, haig
Z. p. haigamchabensis (n = 10)	9.00 ± 5.19	sun, des < haig = atm, vaal > sen, can, vir, cap

Table 2.9: A Discriminant Function Analysis matrix indicating the observed classification percentages using the morphometric data for four southern African *Zosterops* taxa. sen = *Z. senegalensis*, vir = *Z. virens*, cap = *Z. capensis*, pal = *Z. pallidus*.

Taxa	% Correct	sen	vir	cap	pal
sen	86.96	40	4	1	1
vir	89.71	3	244	9	16
cap	11.65	1	70	12	20
pal	62.04	0	44	8	85
Total	**68.28**	**44**	**365**	**29**	**106**

Table 2.10: A Discriminant Function Analysis matrix indicating the observed classification percentages using the combined data for four southern African *Zosterops* taxa. sen = *Z. senegalensis*, vir = *Z. virens*, cap = *Z. capensis*, pal = *Z. pallidus.*

Taxa	**% Correct**	**sen**	**vir**	**cap**	**pal**
sen	91.30	42	4	0	0
vir	99.63	1	271	0	0
cap	100.00	0	0	103	0
pal	97.08	0	3	1	133
Total	**98.38**	**43**	**278**	**104**	**133**

Figure legends

Figure 2.1: Map of southern Africa outlining the distributions of all putative *Zosterops* taxa and areas of overlap, as well as showing the localities of *Zosterops* specimens obtained from museums and collected in the field. The heavy solid line represents the area where *Zosterops* are absent. Adapted from Moreau 1957, Clancey 1967, Skead 1967 and Hockey *et al*. 2005. atm = *Z. v. atmorii*; can = *Z. v. caniviridis*; cap = *Z. v. capensis*; vir = *Z. v. virens*; des = *Z. p. deserticola*; haig = *Z. p. haigamchabensis*; sun = *Z. p. sundevalli*; vaal = *Z. vaalensis*; sen = *Z. senegalensis*.

Figure 2.2: One-way ANCOVA plots of the mean and 95% confidence intervals for a) bill-, b) wing-, and c) tail-length of the nine putative *Zosterops* taxa.

Figure 2.3: Visualization of the grouping of the nine *Zosterops* taxa from multi-dimensional scaling (MDS) analysis of the: a) morphometric, b) quantitative colour*, c) percentage colour and d) combined data sets. S = *Z. senegalensis*, G = Green-bellied (*Z. v. virens* & *Z. v. caniviridis*), C = Grey-bellied (*Z. v. capensis* & *Z. v. atmorii*), V = *Z. vaalensis*, O = Orange River White-eye (*Z. p. sundevalli*, *Z. p. deserticola* & *Z. p. haigamchabensis*). * = only three individuals of *Z. senegalensis* were available for standardized photography for the quantitative colour assessment.

Figure 2.4: A magnified version of the green-bellied grouping of Fig. 2d, indicating the relationship between green bellied Cape White-eye, *Z. virens* and subspecies of *Z. senegalensis*. G = Green bellied (*Z. v. virens* & *Z. v. caniviridis*), V = *Z. vaalensis*, Ska = *Z. s. kasaicus*, Sst = *Z. s. stenocrita*, St = *Z. s. toruensis*, Sp = *Z. s. poensis*, Ssa = *Z. s. kaffensis*, Sr = *Z. s. reichonowi*, Sn = *Z. s. stierlingi*, Sl = *Z. s. demeryi*.

Figure 2.5: Parsimony trees for the continuous (i) and discrete (ii) character analyses: a) morphometric, b) quantitative colour, c) percentage colour and d) combined data set of the nine southern African *Zosterops* taxa. The values on the branches are bootstrap values (1000 psuedoreplicates).

Figure 2.6: One way ANOVA plots of the mean and 95% confidence intervals for the a) red, b) green and c) blue colour score from the throat of the nine putative *Zosterops* taxa.

Figure 2.7: One-way ANOVA plots of the mean and 95% confidence intervals for the a) red, b) green and c) blue colour score from the belly of the nine putative *Zosterops* taxa.

Figure 2.8: One-way ANOVA plots of the mean and 95% confidence intervals for the a) red, b) green and c) blue colour score from the right flank of the nine putative *Zosterops* taxa.

Figure 2.9: One-way ANOVA plots of the mean and 95% confidence intervals for the a) red, b) green and c) blue colour score from the left flank of the nine putative *Zosterops* taxa.

Figure 2.10: One-way ANOVA plots of the mean and 95% confidence intervals for the percentage a) grey, b) green, c) cinnamon, d) yellow and e) white found on the underpart of the nine putative *Zosterops* taxa.

Fig. 2.1

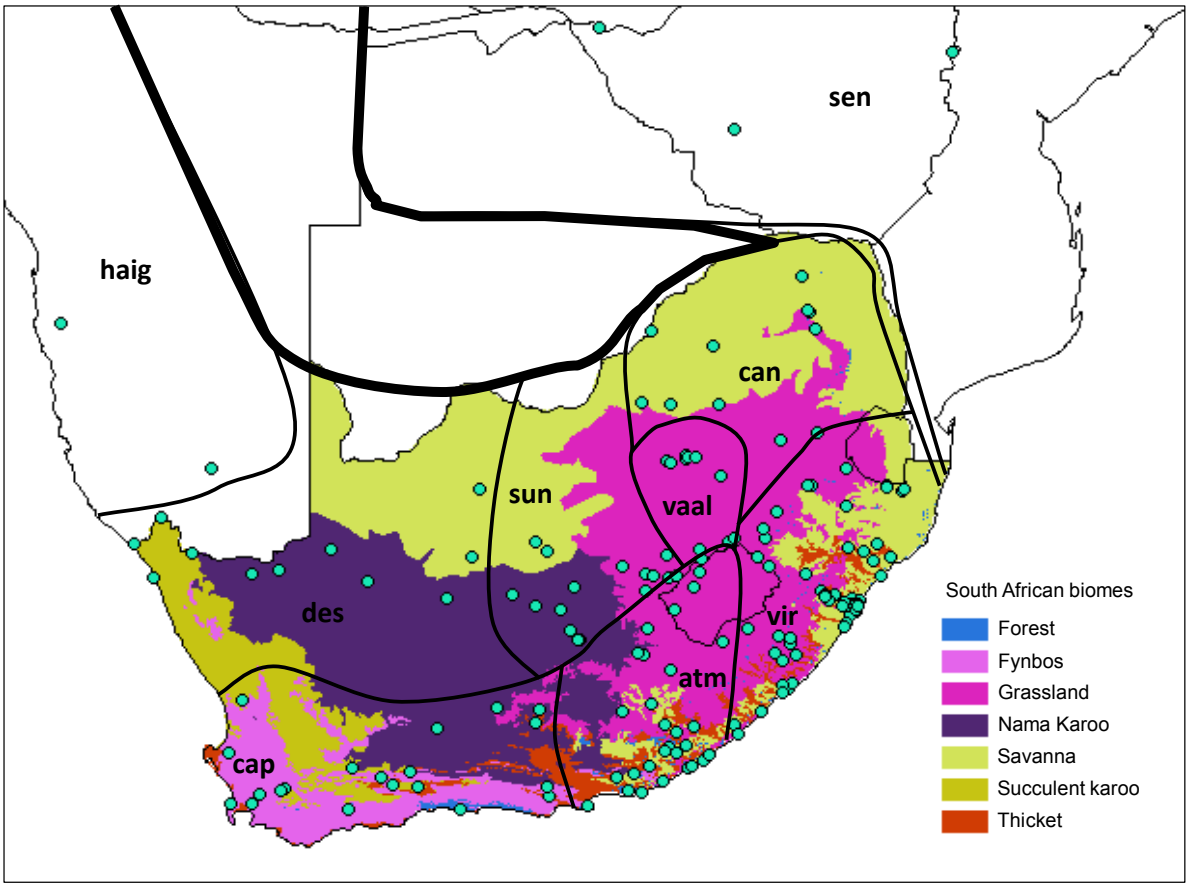


Fig. 2.2

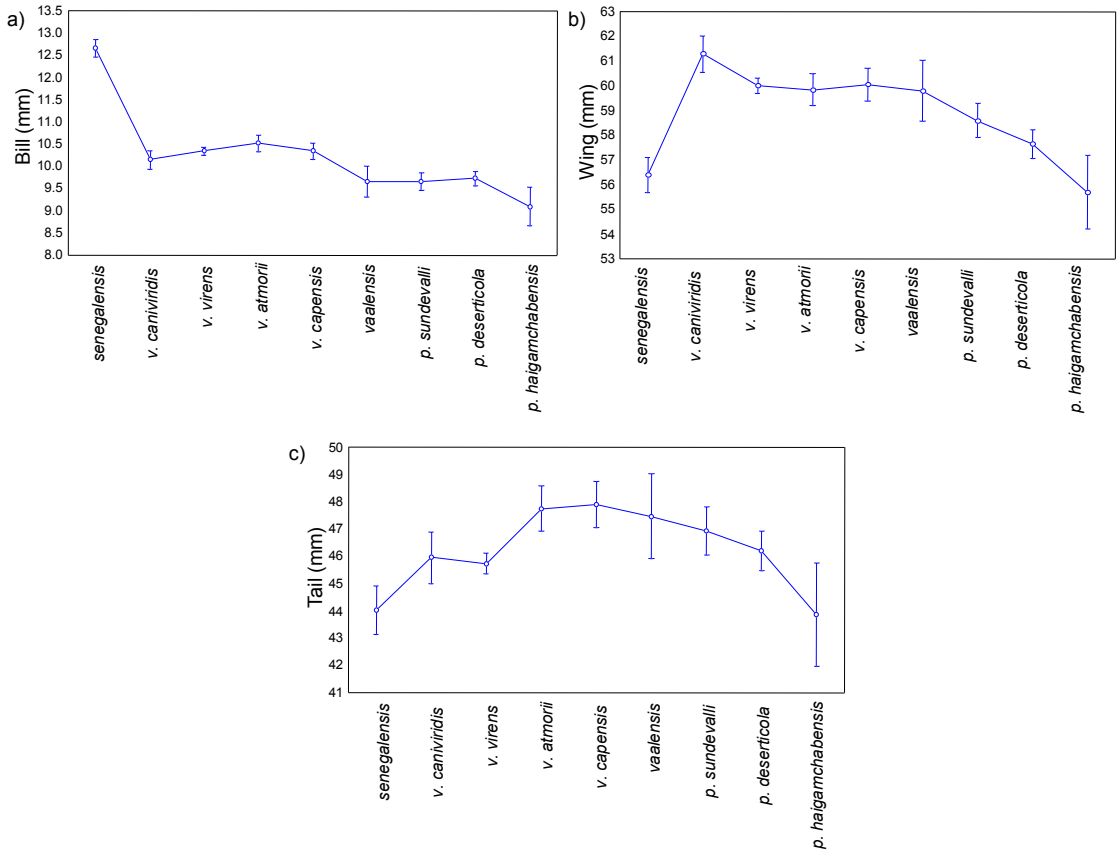


Fig. 2.3

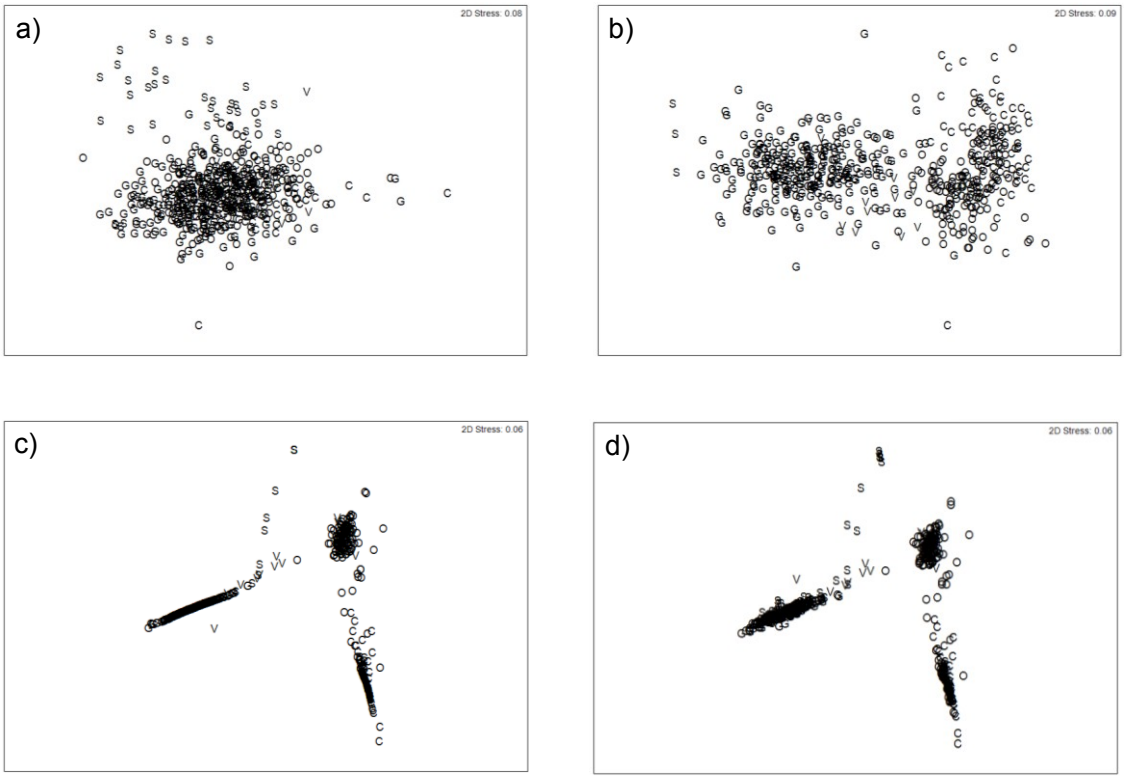

a)
2D Stress: 0.08
b)
2D Stress: 0.09
c)
2D Stress: 0.06
d)
2D Stress: 0.06

Fig. 2.4

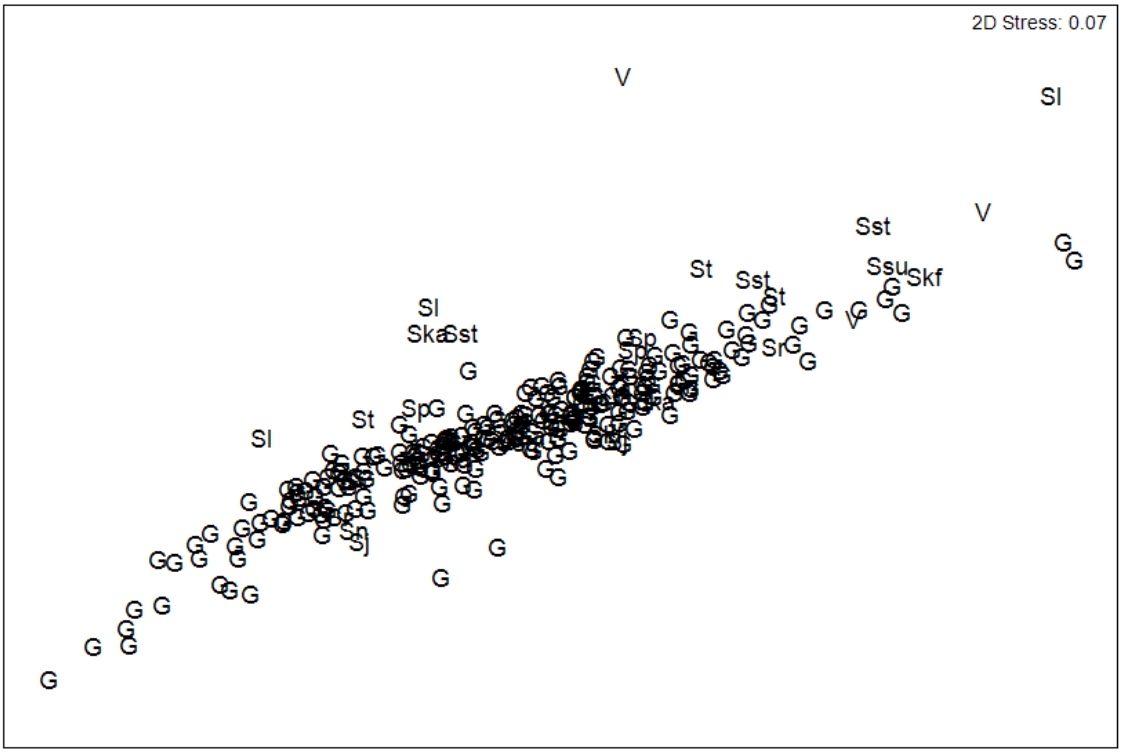

2D Stress: 0.07
V
Sl
V
Sst
St
Ssu
Skf
Sst
St
Sl
Ska
Sst
St
Sl
Sj

Fig. 2.5

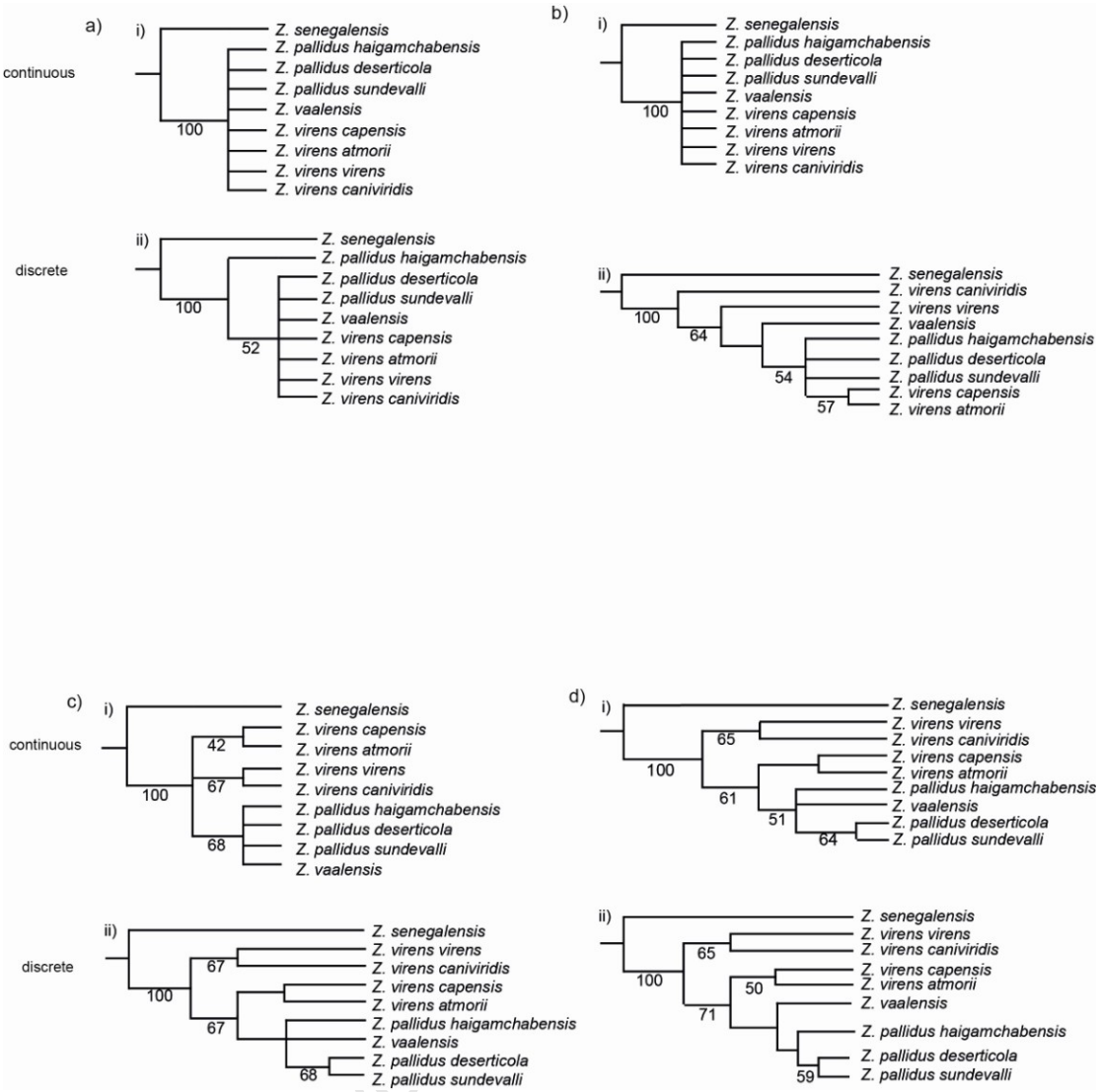

a)
i)
continuous
Z. senegalensis
Z. pallidus haigamchabensis
Z. pallidus deserticola
Z. pallidus sundevalli
Z. vaalensis
Z. virens capensis
Z. virens atmorii
Z. virens virens
Z. virens caniviridis
100
ii)
discrete
100
52
b)
i)
100
ii)
100
64
54
57
c)
i)
continuous
42
67
100
68
ii)
discrete
67
100
67
68
d)
i)
65
100
61
51
64
ii)
65
100
50
71
59

Fig. 2.6

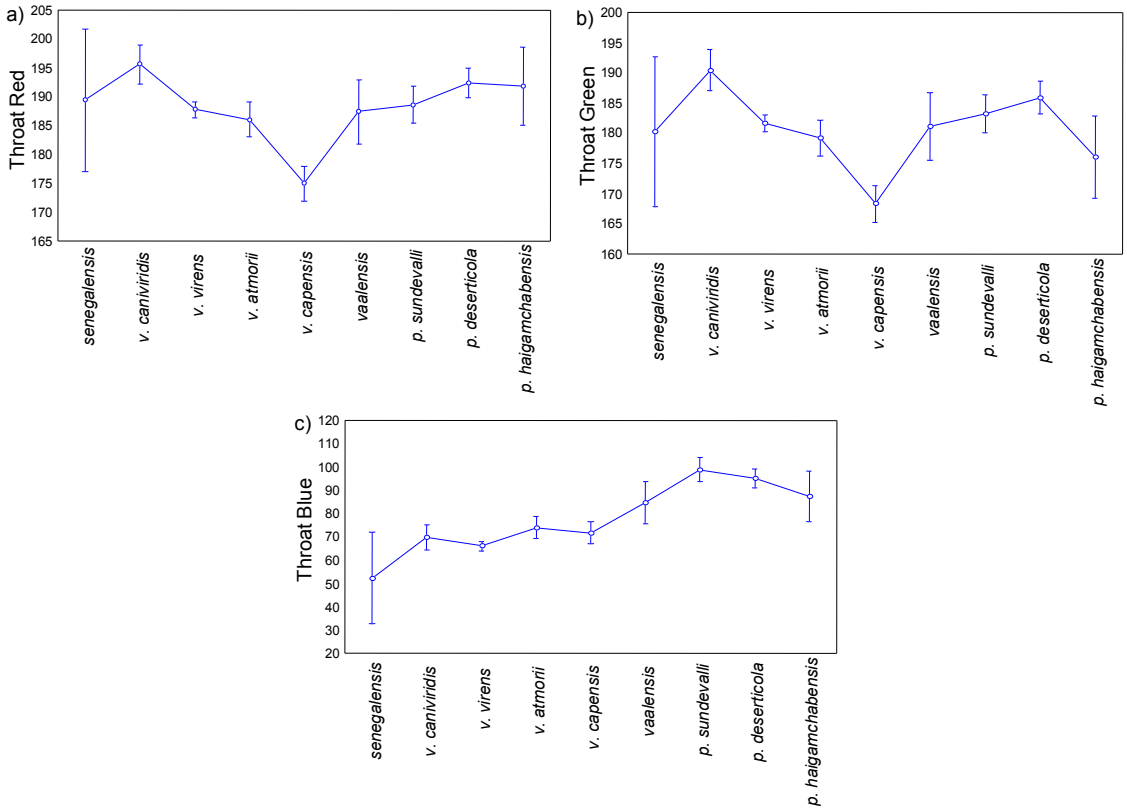


Fig. 2.7

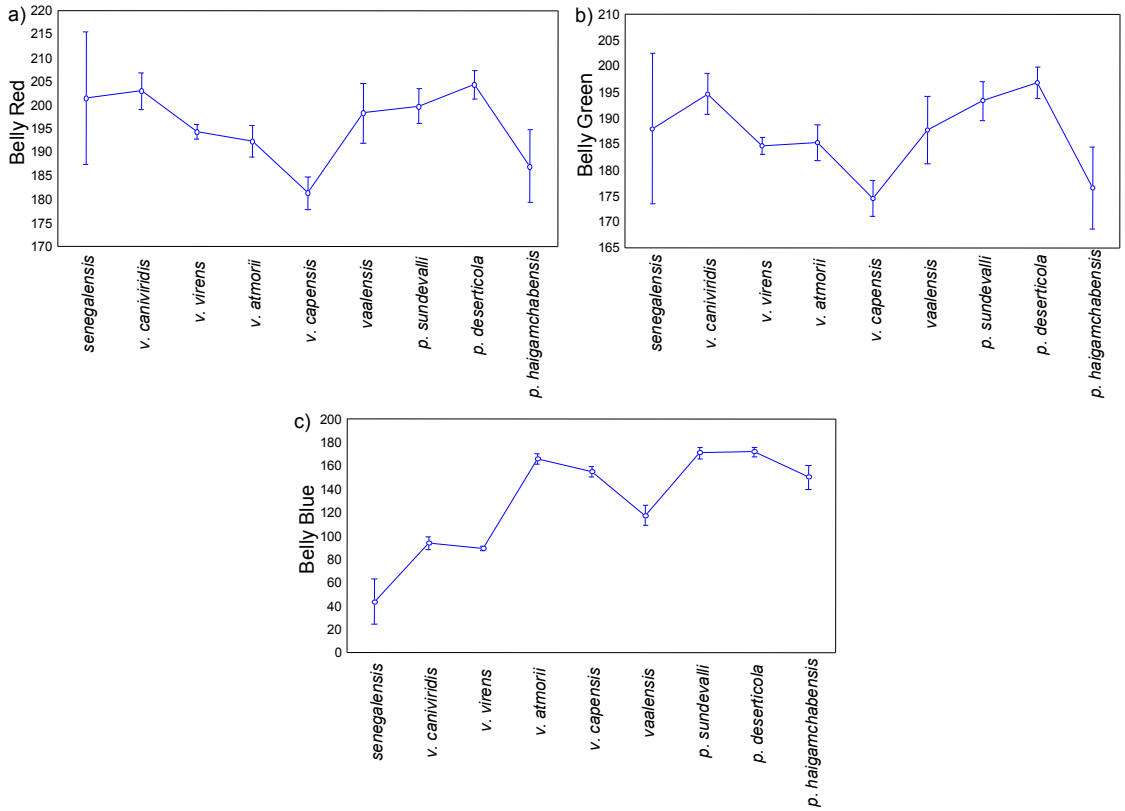


Fig. 2.8

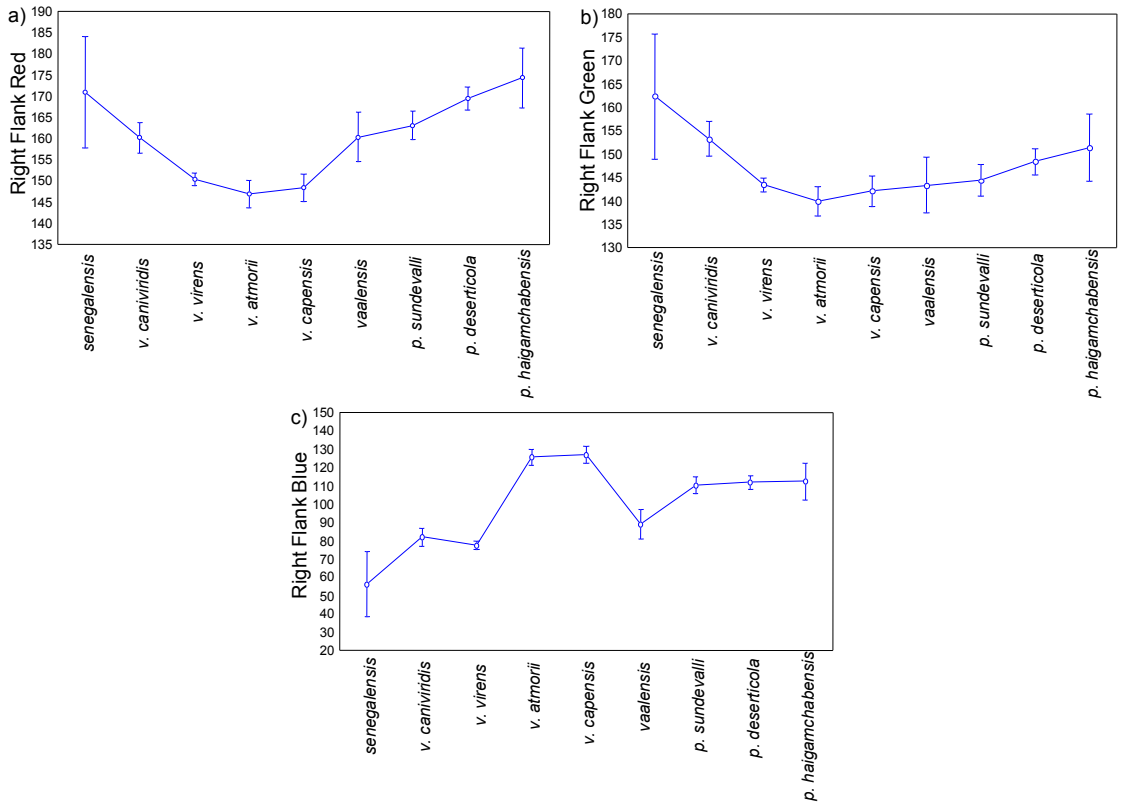


Fig. 2.9

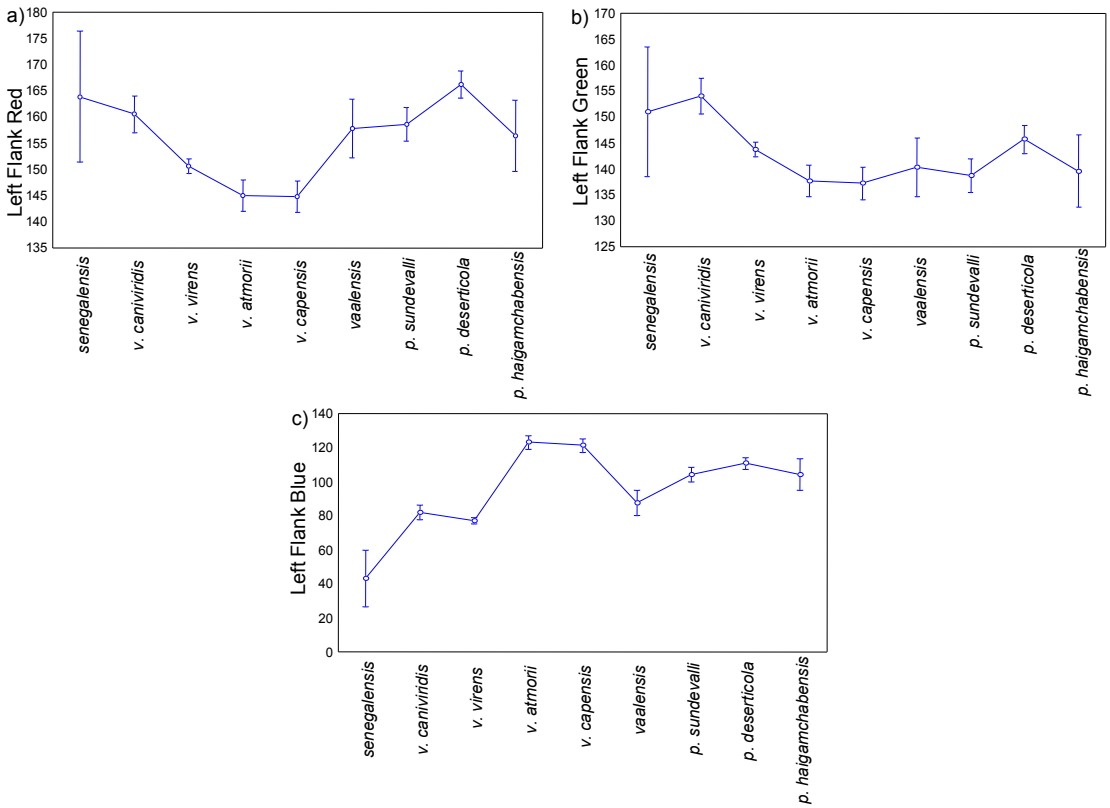
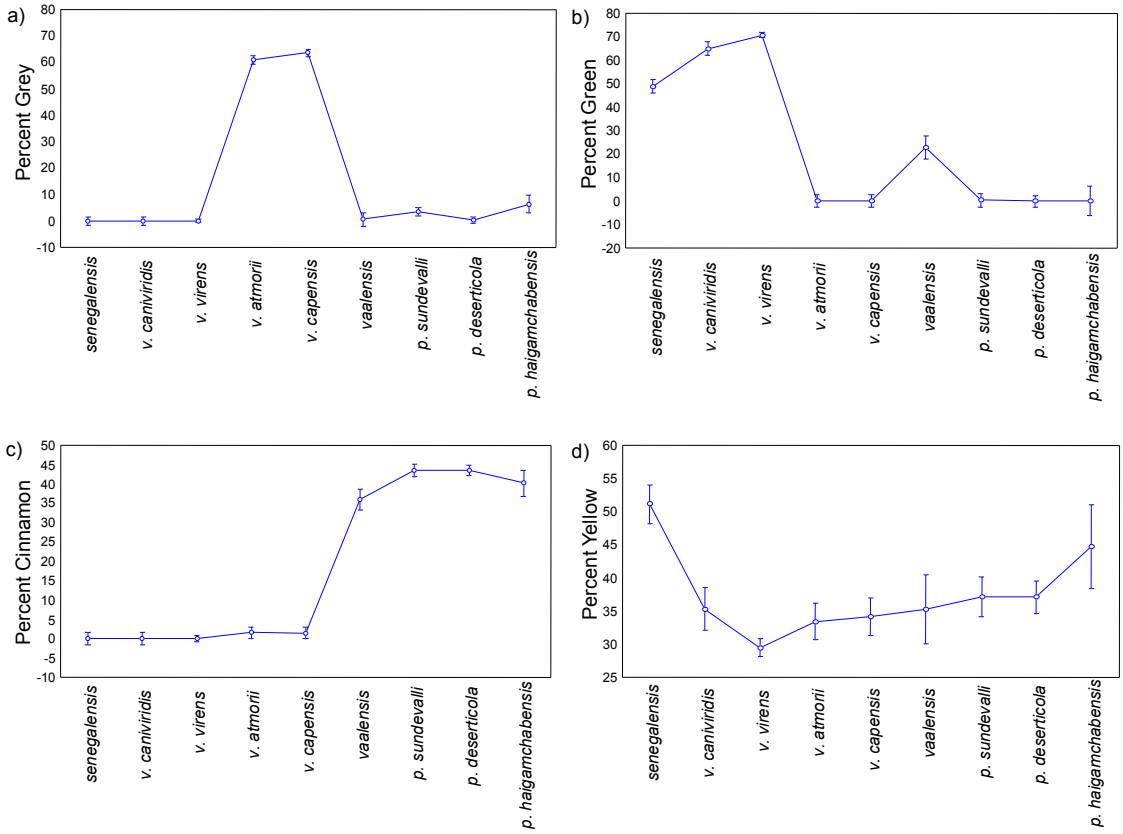


Fig. 2.10



Chapter 3

The utility of song and contact call characters in the systematics of four southern African white-eye species (*Zosterops* spp.)

3.1. Abstract

Bird song and contact calls play different, yet important roles in bird communication and signaling. It is shown here that the use of both vocalization types can lend support to establishing systematic relationships in southern African *Zosterops* taxa. The song data indicate statistically significant differences between the Orange River White-eye *Z. pallidus* and the other three *Zosterops* taxa examined. The variables: 'minimum frequency per song', 'number of elements per song' and 'song length' were the primary discriminatory factors. There are also significant differences in contact calls of *Z. pallidus* and those of hybrid individuals when compared to those of the Grey Cape White-eye *Z. capensis* and the Green Cape White-eye *Z. virens*. Sonogram visualization suggests that 'mean element length' and 'delivery rate' are important characteristics distinguishing contact calls. These results confirm previous anecdotal suggestions of vocal differences between *Z. pallidus* and *Z. capensis*/*virens*. The results also surprisingly suggest that the song of the much more widespread African Yellow White-eye *Z. senegalensis* is very similar to that of both *Z. capensis* and *Z. virens*. The differences in the vocalizations are likely due to contrasting habitat types inhabited by these taxa, with *Z. pallidus* found in the flat, open, arid Succulent Karoo, Nama Karoo and Grassland Biomes where longer vocalizations and more elements per song/call are likely to travel further to conspecifics. The use of hybrids' contact calls also indicates that in areas of overlap between taxa, vocal recognition appears to break down between vocally distinct taxa.

3.2. Introduction

Social contact between populations of a species is necessary to maintain gene flow and cohesion, with the use of vocalizations (contact calls, mating calls and song) often playing an important role when recognizing conspecific individuals. Vicariance events can lead to isolation of populations, which may lead to the divergence of vocal traits between the isolated populations (via selection or drift), that in turn may lead to a lack of recognition during secondary contact (i.e. pre-zygotic barriers) (Slabbekoorn & Smith 2002, Tobias *et al.* 2010b). Differences in environment are also proposed to lead to vocal divergence through the differences in the propagation of signal through differing habitat types (Morton 1975, Dingle *et al.* 2008, Tobias *et al*. 2010b).

Studies of bird vocalizations can be used to address a variety of biological questions, which range from investigating patterns of song learning (Mennill and Rogers 2006) and evolution (e.g. Price and Lanyon 2002, 2004), to investigating dialect variation within species (Baptista 1977, Ritchison 1981, Benedict and Bowie 2009). The use of vocal characters as an informative tool in systematics is on the increase with advances in sonogram methodology (Päckert *et al.* 2003) and analytical software (Charif *et al.* 2006) making it possible to extract highly quantifiable information from each individual's song or call bout. The primary use of vocalizations in systematics has been to help infer the specific status of morphologically similar or cryptic species (e.g. Alström and Ranft 2003, Rheint *et al.* 2008, Toews and Irwin 2008, Alström *et al*. 2011). However, studies have also combined vocal characters with molecular evidence to investigate whether vocal data have any phylogenetic signal (e.g. Päckert *et al.* 2003, Farnsworth and Lovette 2008).

Within passerine birds, the suboscines generally inherit their song, showing no song learning capabilities (Kroodsma 1982, 1984). Oscines however, do show song learning ability (Kroodsma 1982, 1996), with numerous species exhibiting large song repertoires and

mimicry of other birds' song (Catchpole & Slater 2008). Songs, which are characteristically long and complex (Catchpole & Slater 2008), are generally given by males during the breeding season in an attempt to defend territories and attract mates (Kroodsma & Byers 1991, Catchpole & Slater 2008), although females have also been recorded performing songs (see Langmore 1998, Garamszegi *et al*. 2007). In comparison, avian calls are simpler, shorter sounds used by both sexes during the year, that are generally thought not to be subject to learning (Lanyon 1960, Hinde 1970, Marler 2004, Catchpole & Slater 2008), although exceptions are known (Mundinger 1979, Moravec *et al.* 2006).

Cultural song learning can present a major obstacle in using oscine passerine bird song for taxonomic inference. The complex nature of bird song, coupled with oscine learning ability, can result in song plasticity (Krebs and Kroodsma 1980, Mundinger 1982) and mimicry (Kelly *et al.* 2008) which can prove difficult from an analytical perspective. The usefulness of song learning in understanding the processes of speciation between taxa should however, not be overlooked. Changes in song (often quickly) due to either drift or selection may facilitate the formation of pre-zygotic isolating barriers between taxa, giving rise to cases of rapid speciation (e.g. indigobirds *Vidua* spp., Balakrishnan and Sorenson 2006).

This study incorporates vocal characters in the delimitation of species boundaries in southern African white-eyes (*Zosterops* spp.), which has proven difficult in the past (Moreau 1957, Skead 1967, Chapter 2), and thereby aims to provide insight into the pattern of speciation across a heterogeneous landscape encompassing several distinct biomes (Rutherford 2004). Presently, three species of *Zosterops* are recognized in southern Africa (Hockey *et al.* 2005), with four morphologically distinct taxa (Chapter 2). These four white-eye taxa form the operational units recognized for the purpose of this study. Although no comprehensive vocal analyses have been performed on African white-eyes, differences have been noted between *Z. pallidus* (Orange River White-eye) and both *Z. capensis* (Grey Cape

White-eye) and *Z. virens* (Green Cape White-eye) (Skead 1967). There are no apparent differences in vocalization between *Z. virens* and *Z. senegalensis* (African Yellow White-eye) (Skead 1967).

With the importance of vocal communication between conspecifics playing an integral part to species cohesion, differences in vocalizations can form pre-zygotic barriers to mating, leading to speciation between populations with contrasting vocalization types. Here we investigate whether differences in either the songs or calls of morphologically distinct southern African *Zosterops* taxa exist and, if so, could these differences represent species barriers between taxa, or are these vocal differences adaptations to the varying habitats these birds occupy in the region.

3.3. Methods

3.3.1. Vocal sampling

Two types of *Zosterops* vocal communication were obtained in order to assess differences between putative taxa. Songs from all four taxa: *Z. capensis* (n = 4), *Z. virens* (n = 10), *Z. pallidus* (n = 11) and *Z. senegalensis* (n = 6), were obtained from the British Library Sound Archive and from recordings made in the field. Vocalizations obtained from the field were recorded using a Marantz 74CP 130/02B cassette recorder, Sennheiser MKH416T directional microphone and Brodan parabola and microphone system. The same sources as above were used to obtain contact calls from: *Z. capensis* (n = 5), *Z. virens* (n = 3), *Z. pallidus* (n = 5) and several putative hybrid individuals (n = 7). Hybrid individuals were either a mix between *Z. pallidus* and *Z. capensis*, or a mix between *Z. pallidus* and *Z. virens* and identified visually through the inspection of underpart plumage colouration (Chapter 2).

3.3.2. Vocal measurements

The same vocal measurements (see below) were used for analyses of both *Zosterops* songs and contact calls. Because cultural learning is likely to produce complex variation between geographic populations, general characters were chosen for analyses to avoid the potential effects of plasticity in confounding taxonomic inference (Tobias *et al.* 2010a). The following measurements were quantified:

1) song/call duration (s); 2) number of notes per song/call; 3) delivery rate (number of notes/s); 4) maximum frequency per song/call (Hz); 5) minimum frequency per song/call (Hz); 6) mean frequency per song/call (Hz); 7) proportion of no vocal activity, which is the duration of time during which an individual is not calling or singing; 8) mean note frequency change (maximum – minimum frequency) per song/call (Hz); and 9) mean note duration per song/call (s).

The mean frequency per song/call was determined by recording the highest frequency every 0.25s throughout the song/call. Similarly, the proportion of no vocal activity was determined by the number of time points where no vocal activity occurred throughout a song/call. Vocalizations were visualized by producing sonograms in Raven Lite ver. 1.0 (Charif *et al.* 2006), and all measurements were recorded on screen.

3.3.3. Analyses

Mean values for each of the vocal measurements were obtained for each individual and used in all analyses. Measurements that were highly correlated ($r > 0.80$) with other variables were removed from the statistical analyses. For the contact call data set correlated measurements were: number of notes per call and mean note frequency change call, and highly correlated song measurements were: number of notes per song, minimum frequency

per song, proportion of no vocal activity, mean note frequency change per call and mean note length per call.

Differences in vocal characters from each of the vocal data sets (song vs. call) among the four taxa were tested using one-way MANOVA with a post-hoc Tukey test to determine which taxa grouped together using Statistica 9 (StatSoft, Inc. 2004). In order to identify patterns of hierarchical similarity, Euclidean distance was chosen as the similarity coefficient and multi-dimensional scaling analyses were performed on each vocal data set separately using PRIMER 6.1.5 (Plymouth Marine Laboratory, U.K.).

To determine which vocal characters (if any) from each of the two vocal data sets primarily distinguish the four white-eye taxa, *a posteriori* Discriminant Function Analyses were performed using Statistica 9 (StatSoft, Inc. 2004). These results were compared to those of a Principal Components Analysis also performed on each vocal dataset independently.

3.4. Results

3.4.1. Song analyses

All song measures used in the MANOVA calculations showed significant differences among taxa (Wilks Lambda = 0.10, $F_{12, 63.8} = 7.30$, $p < 0.001$). Delivery rate and mean frequency differentiated the Orange River White-eye from the remaining taxa (Tukey test, $p < 0.01$). Maximum frequency also differentiated *Z. pallidus* and *Z. virens* songs (Tukey test, $p = 0.02$), with the length of songs of *Z. pallidus* being significantly longer than *Z. virens* and *Z. senegalensis* songs (Tukey test, $p < 0.001$).

A Discriminant Function Analysis performed on the song data indicated that the main discriminatory characters within the song data set were delivery rate ($p < 0.001$; Partial Lambda = 0.40) and mean frequency ($p = 0.005$; Partial Lambda = 0.59). The first principal component of the song data accounted for 83.2% of the variation, with mean frequency and

song length explaining most of this variation (Table 3.3). Two groupings emerged from the multi-dimensional scaling analyses; the first composed of *Z. pallidus* songs, whereas *Z. capensis*, *Z. virens* and *Z. senegalensis* songs made up the second group (Fig. 3.3a).

3.4.2. Call analyses

Calls were recorded during all seasons, and are probably used to maintain contact between individuals within pairs or among individuals forming part of a feeding flock. The structure of *Z. pallidus* and hybrid individuals differed with those of *Z. capensis* and *Z. virens* calls (Fig. 3.2). Calls of *Z. pallidus* are made up of groups of two or three short notes (mean = 0.047 s; Fig. 3.2d), with contact calls of both *Z. capensis* and *Z. virens* are composed of single, longer notes (mean = 0.187 s and 0.206 s respectively; Fig. 3.2a and 3.2b). The smooth, parabola-like shape of *Z. pallidus* contact call notes (Fig. 3.2d) are visible in some hybrid notes (Fig. 3.2c), whereas other notes show the more ragged characteristics of *Z. capensis* and *Z. virens* notes (Fig. 3.2a and 3.2b).

Significant differences were observed among taxa (MANOVA Wilks lambda = 0.48, $F_{29,\,29.3} = 2.62$, p = 0.008) with delivery rate explaining differences between *Z. pallidus* and both *Z. capensis* and *Z. virens* (Tukey test, p = 0.017 and 0.011, respectively). Mean note length per note differentiated *Z. capensis* and *Z. virens* from both *Z. pallidus* and hybrid individuals (Tukey test, p < 0.001). This measure was also identified as the main discriminatory character using a Discriminant Functions Analysis (p = 0.006, Partial Lambda = 0.30). Three *Z. capensis* individuals separate from the remaining white-eye calls and have a shorter duration (Fig. 3). Differences in delivery rate appear to distinguish *Z. pallidus*, *Z. capensis* and *Z. virens*, with hybrid individuals intermediate between these groups (Table 3.2, Fig. 3.3).

3.5. Discussion

Vocalizations are import forms of communication for intraspecific genetic cohesion and interspecifically for the maintenance of species boundaries (i.e. pre-zygotic isolation). Although more complex than contact calls and formed through learning in oscine passerines, song can be used as a taxonomic indicator if broad vocal measures/characters are used in evolutionary inference. This study has incorporated both song and contact calls of southern African white-eye taxa to help elucidate relationships among these taxa.

Although never investigated fully in the past, it has been reported that the vocalizations (both song and call) of *Z. pallidus* are different from those of *Z. capensis* and *Z. virens* (Skead 1967). Quantitative analysis of white-eye songs has demonstrated that this indeed appears to be the case, with significant differences reported for the four uncorrelated song characters. In all these cases, *Z. pallidus* was readily distinguished from the remaining three taxa (Tukey test, $P < 0.01$). The MDS plot of the song data (Fig. 3.3a) illustrates the distinction between *Z. pallidus* and the remaining taxa, but does not indicate any separate groupings of *Z. capensis*, *Z. virens* and *Z. senegalensis*. This lack of grouping among these groups is somewhat surprising. Striking plumage colour, and to a lesser extent morphometric, differences (Chapter 2), coupled with the large geographic area that these taxa cover (northwest, eastern and southern Africa) and a diversity of habitat types (forest vs. savanna vs. fynbos) that these birds occupy would be expected to provide opportunities for song divergence in these oscine passerines where song learning and mimicry are common (Skead 1967, Cohen and Winter 1992, Fry *et al*. 2000). The lack of variation observed here may be due to physiological similarities (i.e. syrinx morphology) or genes controlling the production of particular sounds in juvenile birds (Podos *et al*. 2004).

Taxonomic inferences can be made from the contact call data. Visual sonogram inspection (Fig. 3.2) shows the fundamental differences between *Z. pallidus* and both *Z.*

capensis and *Z. virens* contact calls, with statistical significance obtained for values of delivery rate and mean note length per call. The increased number of shorter notes and speed of the call delivery are the primary factors contributing to the anecdotal differences noted by C.J. Skead in his book of southern African white-eyes (1967). Hybrid individuals produce intermediate calls, grouping together *Z. pallidus* and both *Z. capensis* and *Z. virens* individuals in multidimensional space (Fig. 3.3b). Visual inspection of hybrid sonograms (Fig. 3.2c) highlights the intermediate character of hybrid contact calls. Contact calls of *Z. pallidus* are made up of groups of two or three short notes (mean = 0.047 s; Fig. 2d), with contact calls of both *Z. capensis* and *Z. virens* composed of a single longer note (mean = 0.187 s and 0.206 s respectively; Fig. 3.2a and 3.2b). The smooth, parabola-like shape of *Z. pallidus* contact call notes (Fig. 3.2d) are visible in some hybrid notes (Fig. 3.2c), whereas other notes show the more ragged characteristics of *Z. capensis* and *Z. virens* notes (Fig. 3.2a and 3.2b). Further, although not significantly different, the mean note length of hybrid individual calls is consistently longer than that of *Z. pallidus* (Table 3.2).

Multiple factors are likely to drive the observed vocal differences observed between *Z. pallidus* and the other *Zosterops* taxa. Morphology is predicted to have an influence on vocalization production, with larger organisms expected to produce sounds of lower frequency (Ryan and Brenowitz 1985). However, *Z. pallidus* is similar in size to *Z. capensis* and *Z. virens*, and only slightly larger than *Z. senegalensis* (Chapter 2). Although there is a significant difference in mean frequency per song, this difference does not correspond to expected change in size where the smaller *Z. senegalensis* would be expected to have a higher mean frequency than the other taxa.

The similarity of both song and call variation suggests that a single factor (Irwin *et al.* 2008) may be behind the formation of white-eye vocalization. Physiological and neurological processes play a role in vocalization production (Marler 2004), thus at a proximate level it is

plausible that a different syrinx shape or changes in neurological pathways may explain the observed pattern of song and call differences between *Z. pallidus* and the other white-eye taxa.

Ultimately though, the song and call differences observed here are likely to have arisen through differential adaptations to habitat. The core distribution of *Z. pallidus* incorporates the arid biomes of southern Africa, mainly northwestern South Africa, extending across the Orange River into Namibia (Moreau 1957, Clancey 1967, Skead 1967, Hockey *et al.* 2005). Although occurring mainly in the wooded vegetation along river watercourses, the predominant biome types of *Z. pallidus* distribution are the Succulent and Nama Karoo, and Grassland Biomes. The Succulent Karoo is dominated by low to dwarf shrubs (Milton *et al.* 2004), and the Nama Karoo by a combination of succulent shrubs, arid-adapted grasses and annuals (Palmer and Hoffman *et al.* 2004). Habitat has been shown to affect vocalizations in bird species (Badyaev and Leaf, 1997, Dingle *et al.* 2008, Tobias *et al.* 2010b), with taxa from open habitats such as grasslands exhibiting more rapid, higher frequency sounds (Morton 1975). Together with the grasslands of the Free State, the Succulent and Nama Karoo are both flat, open environments, and it could therefore be expected that similar vocal patterns would be found in birds inhabiting these biomes. In contrast, *Z. virens* inhabits dense forest and coastal shrub, and *Z. capensis* fynbos tickets and riverine forest.

The intermediate composition of contact calls of hybrid individuals indicates that components of parental species' calls are being expressed in offspring from interbreeding species. Contact calls are thought not to be a learned behaviour, and be heritable (Marler 2004), an expectation which is consistent with our results. Further investigation of hybrid zone dynamics among southern African *Zosterops* taxa, including playback experiments within hybrid zones, could reveal which factors are important in white-eye species recognition.

Table 3.1: Mean ± standard deviation of song characters for *Z. capensis*, *Z. virens*, *Z. pallidus* and *Z. senegalensis*.

Character	*capensis* (4)	*virens* (10)	*pallidus* (11)	*senegalensis* (6)
song length	5.48 ± 4.00	3.95 ± 1.02	7.86 ± 1.71	3.25 ± 1.41
No. elements per song	27.04 ± 19.14	15.14 ± 4.08	52.57 ± 14.50	14.48 ± 3.98
Delivery rate (no. ele/s)	4.98 ± 0.69	3.84 ± 0.27	6.70 ± 0.72	4.85 ± 1.53
Minimum frequency per song (Hz)	1416.06 ± 167.29	1562.89 ± 229.30	887.86 ± 108.91	1735.63 ± 183.66
Maximum frequency per song (Hz)	6838.53 ± 733.76	6509.49 ± 952.36	7998.82 ± 788.17	6486.78 ± 1820.41
Mean frequency per song (Hz)	2687.72 ± 594.68	2380.23 ± 233.08	1803.88 ± 222.94	2673.68 ± 401.45
Proportion of no vocal activity	29.60 ± 12.68	33.05 ± 7.07	50.99 ± 5.00	29.67 ± 9.04
Mean element frequency change per song (Hz)	2628.23 ± 206.09	2631.99 ± 189.87	3324.04 ± 469.04	2615.33 ± 508.42
Mean element length per song (s)	0.139 ± 0.019	0.171 ± 0.007	0.077 ± 0.007	0.154 ± 0.039

Table 3.2: Mean ± standard deviation of contact call characters for *Z. capensis*, *Z. virens*, *Z. pallidus* and hybrid individuals.

Character	*capensis* (5)	*virens* (3)	*pallidus* (5)	hybrid (7)
call length	7.89 ± 6.74	13.77 ± 4.64	12.75 ± 4.51	9.88 ± 2.98
No. elements per call	8.20 ± 4.97	10.97 ± 2.35	46.10 ± 37.17	19.17 ± 9.99
Delivery rate (no. ele/s)	1.34 ± 0.65	0.83 ± 0.12	3.49 ± 1.38	1.98 ± 1.05
Minimum frequency per call (Hz)	1840.67 ± 232.33	1769.50 ± 232.93	1764.85 ± 139.36	1672.14 ± 93.38
Maximum frequency per call (Hz)	5110.90 ± 438.21	5160.43 ± 492.08	5003.10 ± 370.63	4745 ± 499.83
Mean frequency per call (Hz)	913.20 ± 477.63	620.51 ± 39.38	668.34 ± 224.31	471.65 ± 58.04
Proportion of no vocal activity	73.73 ± 13.47	82.12 ± 1.07	80.12 ± 5.89	85.50 ± 2.82
Mean element frequency change per call (Hz)	2597.98 ± 321.72	2515.57 ± 440.82	2133.79 ± 159.16	2046.29 ± 398.30
Mean element length per call (s)	0.187 ± 0.028	0.206 ± 0.044	0.047 ± 0.009	0.083 ± 0.031

Table 3.3: Eigenvalues, variance explained, and factor loadings of the first three principal components produced in the PCA of song characters.

	PC1	PC2	PC3
Eigenvalue	6.71	1.01	0.34
Cumulative Variance explained	83.2%	95.8%	100%
Factor loading:			
Song length	0.679	0.490	0.220
Delivery rate	0.186	-0.833	0.145
Max freq	-0.179	0.114	-0.840
Mean freq	-0.687	0.229	0.475

Table 3.4: Eigenvalues, variance explained, and factor loadings of the first three principal components produced in the PCA of call characters.

	PC1	PC2	PC3
Eigenvalue	3.25	1.85	0.981
Cumulative Variance explained	52.4%	82.3%	98.1%
Factor loading:			
Call length	0.829	-0.096	0.347
Delivery rate	0.054	0.861	-0.329
Min freq	-0.205	-0.259	-0.199
Max freq	-0.197	-0.274	-0.225
Mean freq	-0.475	0.144	0.710
Prop of no vocal activity	0.033	-0.274	-0.408
Mean note length	-0.039	-0.102	0.105

Figure legends

Figure 3.1: Sonogram visualization of the songs of southern African *Zosterops* taxa: a) Z. *senegalensis*, b) *Z. virens*, c), and *Z. capensis* d) *Z. pallidus*. Sonogram images were produced in Raven Lite ver 1.0.

Figure 3.2: Sonogram visualization of the contact calls of southern African *Zosterops* taxa: a) *Z. capensis*, b) *Z. virens*, c) hybrid, and d) *Z. pallidus*. Sonogram images were produced in Raven Lite ver 1.0.

Figure 3.3: Multi-dimensional scaling plots showing the groupings of *Zosterops* using a) song character data and b) contact call character data. Key: c = *Z. capensis*, v = *Z. virens*, s = *Z. senegalensis*, p = *Z. pallidus*, h = hybrid.

Fig. 3.1

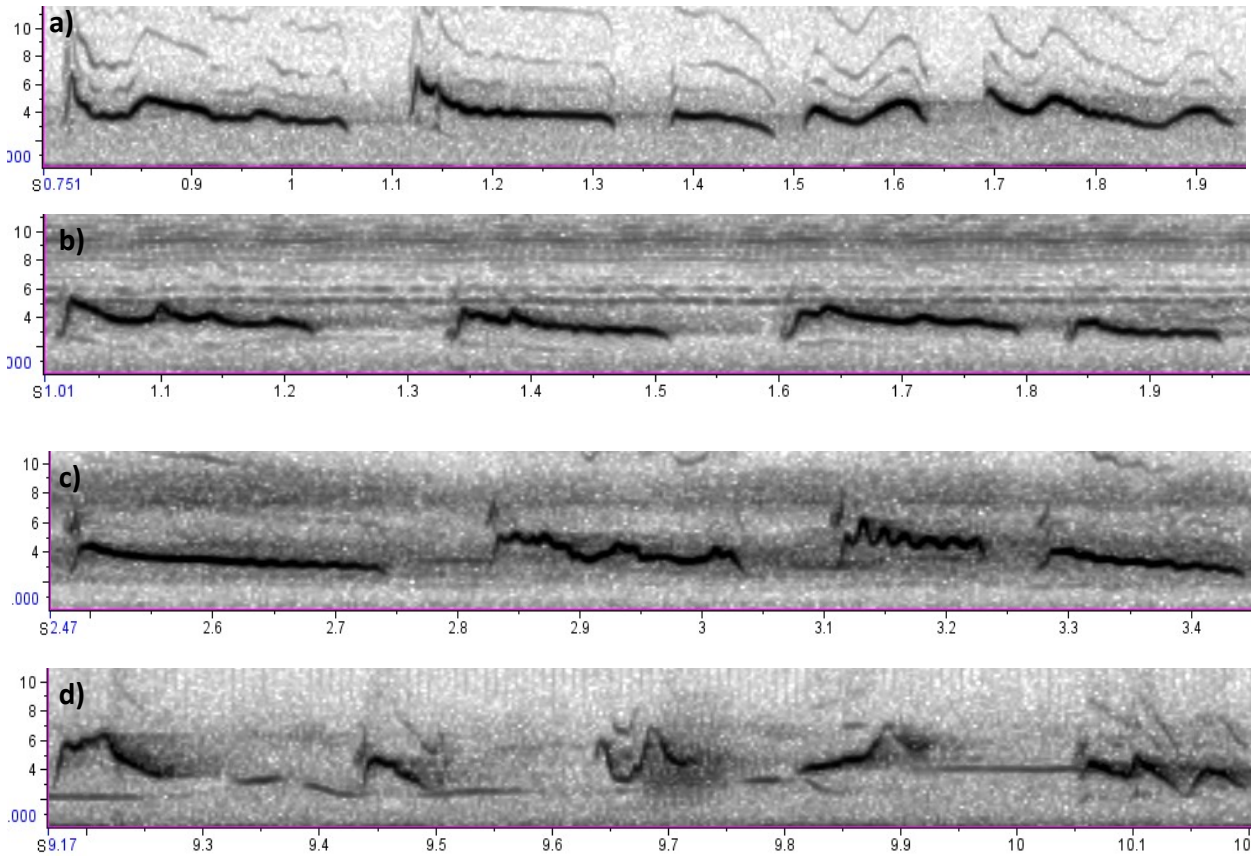


Fig. 3.2

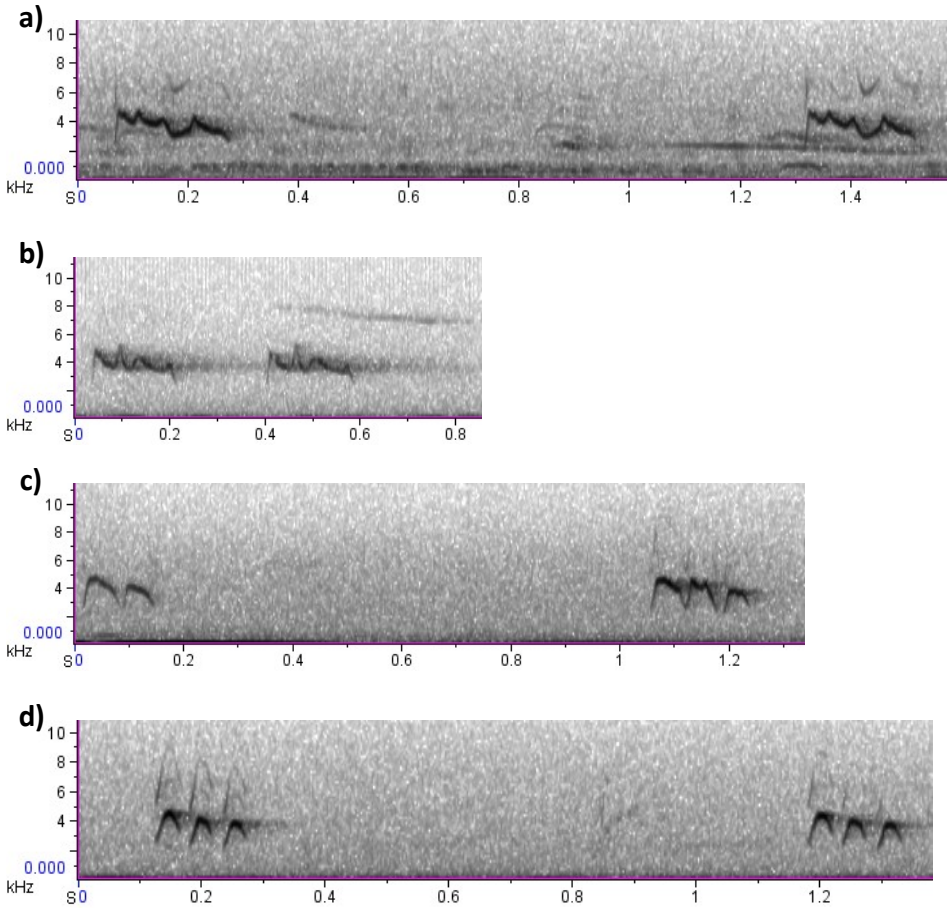
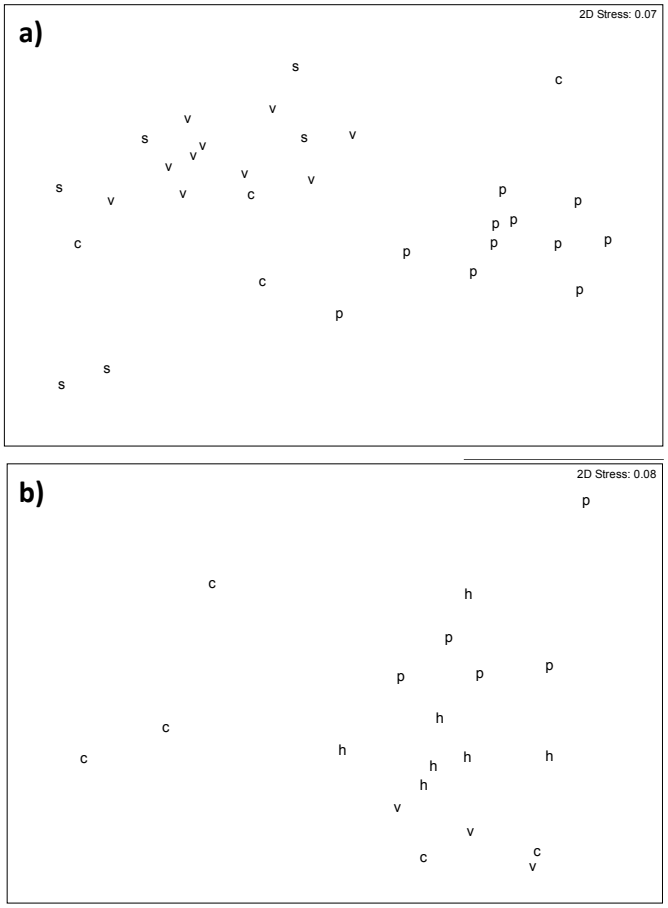


Fig. 3.3



Chapter 4

A multi-locus phylogeny for southern African White-eye *Zosterops* taxa indicates interbreeding between non-sister taxa

4.1. Abstract

The recent, rapid radiation of Zosteropidae, coupled with their high levels of colonizing ability and phenotypic plasticity, makes species delimitation within this family problematic. Given these problems, challenges to establish the mechanisms driving diversity and speciation within this group have arisen. Four morphologically distinct southern African *Zosterops* taxa, with a contentious taxonomic past, provides such a challenge. Here, supplemented with morphological and environmental analytical techniques, a combination of mitochondrial and nuclear markers are analyzed using Bayesian and Likelihood methods to determine their speciation patterns and to establish the systematic relationships of these four morphologically diverse southern African *Zosterops* taxa. Nearly all individuals are phenotypically diagnosable, even those individuals collected in areas of contact between taxa, whereas contact localities appear to possess intermediate environmental characteristics. Initial Bayesian and Likelihood mitochondrial DNA analyses and Bayesian structure analyses of the combined nuclear markers indicated levels of hybridization in contact areas. Subsequently, a combined mtDNA and nuDNA and species tree analysis with hybrid individuals removed placed *Z. pallidus* as basal to the other southern African taxa, with *Z. senegalensis* sister to a clade comprised of *Z. capensis* and *Z. virens*. A dating analysis also provides estimates of divergence for these taxa. The grouping of taxon-specific sampling localities and the apparent intermediate nature of contact sampling localities points toward an influence of habitat type and the associated climatic conditions in driving their plumage diversification in southern Africa.

4.2. Introduction

The rapid and recent radiation of the Zosteropidae (White-eyes), which exhibit one of the highest diversification rates among vertebrates (Moyle *et al*. 2009), makes it both difficult and fascinating to unravel the underlying mechanisms which have led to this remarkable radiation of passerine birds. The high colonization potential of members of the Zosteropidae has resulted in numerous island forms (van Balen 2008), that have undergone rapid morphological change associated with the exploitation of novel habitats and environments, a remarkable contrast to the more typical pattern following divergence of niche conservatism seen in tropical vertebrates (Wiens and Graham 2005, Cadena *et al*. 2011). The pace at which morphological characters, and in particular plumage, appears to change in this complex of birds has led to considerable taxonomic instability, especially with regard to species delimitation (Mees 1953, Moreau 1957, Mayr 1965, van Balen 2008). However, such phenotypic variation provides a wonderful opportunity with which to explore and attempt to understand the patterns and processes of speciation in birds (e.g. Warren *et al.* 2006, Clegg *et al.* 2008, Moyle *et al*. 2009, Clegg and Phillimore 2010, Melo *et al*. 2011).

There have been several attempts to classify and describe the white-eyes found in southern Africa (see Gill 1936, Roberts 1942, Moreau 1957, Clancey 1967, Skead 1967, Hockey *et al.* 2005, van Balen 2008, Chapter 2 and 3). The two primary contributors to *Zosterops* taxonomy in southern Africa (Moreau 1957, Clancey 1967) had contrasting ideas. Moreau (1957) recognized three species, *Z. pallidus*, *Z. senegalensis* and *Z. virens*. Within *Z. virens* he recognized three subspecies, two grey bellied forms (*Z. v. capensis* and *Z. v. atmorii*), and one green bellied form (*Z. v. virens*). Clancey (1967) on the other hand recognized *Z. senegalensis* and lumped *Z. pallidus* and *Z. virens* (including the grey- and green-bellied subspecies) as a single species, giving subspecies status to the three colour forms: *Z. pallidus pallidus* (cinnamon flanks), *Z. p. capensis* (grey-bellied) and *Z. p. virens*

(green-bellied). There is little size difference between *Z. pallidus*, *Z. capensis* and *Z. virens* with these three taxa primarily varying in underpart colouration, which has in the past been the main character used to define the taxonomy of southern African *Zosterops* (Chapter 2). Underpart plumage colouration correlates strongly with the primary habitat that each taxon occupies (*Z. pallidus* – semi-arid shrublands (Karoo), *Z. capensis* – fynbos, *Z. virens* – forest) (Chapter 2). Thus, the spatial or eco-geographic association may have played a prominent role in the regional diversification observed among these taxa.

The African Yellow White-eye (*Zosterops senegalensis*) has never been thought to be conspecific with any of the other southern African taxa. *Zosterops senegalensis* (consisting of multiple subspecies) ranges from eastern Kwa-Zulu Natal and Mpumalanga, South Africa, northwards into East and West Africa (Moreau 1957) and is ecologically separated from *Z. virens,* preferring the deciduous bush of the low-veld over the evergreen forests of coastal acacia (Moreau 1957). Morphologically, *Z. senegalensis* has predominantly yellow underparts and has longer tarsi and bill (Chapter 2).

Mitochondrial DNA (mtDNA) has long been the primary marker used in animal molecular taxonomic and phylogeographic research (Avise 2000) due to its lack of recombination (Birky 2001), amplification ease and maternal inheritance (Moore 1995, Ballard and Whitlock 2004). However, mtDNA loci can be under selection (e.g. Cheviron and Brumfield 2009, Ribeiro *et al* 2011) and therefore may not always be the neutral marker sought for phylogenetic analyses. Further, introgression between closely related taxa may mask their true evolutionary history (Ballard and Whitlock 2004). The addition of nuclear DNA (nuDNA) markers provides additional data with which to test phylogenetic relationships among taxa. For instance, when attempting to estimate a species phylogeny, one needs to be aware of possible discordant phylogenies obtained from gene histories (e.g. Jennings and Edwards 2005). Thus, the use of a single locus in estimating a species

phylogeny can be unreliable (Maddison 1997, Nichols 2001, Edwards 2009). Although the mtDNA gene tree will often be correct (Zink and Barrowclough 2008), several processes can lead to discordance among the mtDNA and nuDNA gene trees, and the species tree, including horizontal gene transfer, incomplete lineage sorting, gene duplication, hybridization and recombination (Maddison 1997, Degnan *et al*. 2009, Edwards 2009). In an effort to correctly estimate a true species tree (a tree representing the ancestor-descendent relationships of taxa), several new multi-locus methodology approaches have been developed (Knowles and Kubatko 2010, Heled and Drummond 2010).

This study aims to determine if the morphological diversity observed in southern African *Zosterops*, and the occupation of contrasting environmental conditions by the four regional morphospecies (*Z. pallidus*, *Z. virens*, *Z. capensis*, *Z. senegalensis*, *sensu* Chapter 2), are reflective of the underlying species tree, or indicative of ecotypic variation in response to adaptation to novel habitats. By comparing the evolutionary history of these taxa, inferred with the use of mitochondrial and nuclear genetic data, with other lines of evidence (e.g. morphology, environmental variables and vocalizations), the processes and patterns shaping the diversification of *Zosterops* taxa in the southern African subregion can be determined. Secondarily, the topographical arrangement of taxa in our species tree and associated dating analyses can provide insight into the timing of southward colonization routes of *Zosterops* in Africa given their Asian origin (Moyle *et al*. 2009).

4.3. Material and methods

4.3.1. Sampling

Multiple individuals of each of the four southern African *Zosterops* morphospecies (Chapter 2) were sampled from throughout their respective regional distribution ranges (Table 4.1, Fig. 4.1). Four localities (Aliwal North, Nova Vita, Taba Nchu and Tarkastad) of

putative sympatry based on the distribution of morphological characters (Chapter 2) were included in the sampling scheme. Given that *Zosterops* taxa are thought to have colonised Africa from a single Asian invasion (Warren *et al*. 2006, Moyle *et al*. 2009, Melo *et al*. 2011) two Asian taxa were selected as outgroups: Ranongga White-eye *Zosterops splendidus* and Chestnut-flanked White-eye *Z. erythropleurus*.

4.3.2. DNA extraction and PCR-amplification

Genomic DNA was extracted from tissues using either the standard phenol:chloroform extraction method or by using a DNEASY Kit following the manufacture's recommendations (Qiagen, Valencia, California). Five molecular markers were used: mtDNA ATP Synthase subunit 6 with a small flanking region of Cytochrome Oxidase subunit 3 (ATP6: 714 bp), the autosomal introns Glyceraldehyde-3-phosphate Dehydrogenase intron 11 (GAPDH: 329 bp) and Transformation-growth-factor Beta 2 intron 5 (TGFβ2: 582 bp), as well as two sex-linked loci on the avian Z-chromosome, Chromo-Helicase-DNA binding gene (CHD1: 476bp) and Muscle Skeleton Receptor Tyrosine Kinase (MUSK: 571 bp).

Double stranded DNA templates were amplified by the polymerase chain reaction (PCR). ATP6 was PCR-amplified using a standard protocol for 36 cycles at an annealing temperature of 54°C; GAPDH was amplified over 37 cycles with an annealing temperature of 64°C, and TGFβ2 was amplified over 35 cycles at an annealing temperature of 58°C. The sex-linked loci, CHD1 and MUSK, were both PCR-amplified over 35 cycles at 50°C and 52°C, respectively. The primer sequences used in the PCRs for each of the five markers are detailed in Table 4.2. The PCR cycling was performed by a GENEAMP PCR System 2700 (Applied Biosystems).

Three micro-litres of PCR product was separated and visualized on a 1% Agarose gel, stained with ethidium bromide. The remaining PCR product was purified using 2.5ul of

diluted ExoSAP solution incubated for 30 min at 37°C, and then for 15 min at 80°C. All purified PCR products were cycle-sequenced using Big Dye terminator chemistry (Applied Biosystems, Foster City, CA, USA). Sequenced products were purified using Sephadex columns and then run on an AB3100 or AB3730 automated sequencer. All sequences were checked and edited using BIOEDIT version 7.0.5.3 (Hall 1999). Heterozygous sites in nuclear loci (double peaks) were coded using the appropriate IUPAC code. Alignment was performed by eye in BIOEDIT and was straightforward owing to the low number of insertion and deletion events.

4.3.3. Phylogenetic analyses – mitochondrial DNA

Bayesian and Maximum Likelihood analyses were performed on the mitochondrial DNA (ATP6). The best-fitting model for the Bayesian analysis under the Akaike Information Criterion was identified in MRMODELTEST, version 2.2 (Nylander 2004). Bayesian analyses were performed using MRBAYES version 3.1.2 (Huelsenbeck & Ronquist 2001). Bayesian analyses were performed on partitioned (by codon) and non-partitioned data and compared using Bayes factor (Nylander *et al.* 2004). Two searches were performed for each analysis, using four chains and run for 5 x 10^6 generations, sampling every 1 x10^3 generations. Stationarity was achieved within the first 10% of the generations, thus the burn-in was set at 500. We ensured that the potential scale reduction factor (PSRF) approached 1.0 for all parameters and that the average standard deviation of split frequencies converged towards zero. The two MRBAYES runs were combined to produce the final tree and posterior probabilities. We also used TRACER v1.5 (Rambaut and Drummond, 2007) to ensure that our sampling of the posterior distribution had reached a sufficient effective sample size (ESS) for meaningful parameter estimation.

Maximum likelihood phylogenetic analysis was conducted using RAXML (Stamatakis 2006) partitioned by codon position, under a general-time-reversible model of nucleotide substitution and a gamma model of rate heterogeneity via the CIPRES PORTAL (Stamatakis *et al.* 2008, Miller *et al.* 2009). One-thousand bootstrap pseudoreplicates were performed to evaluate support at specific nodes. A statistical parsimony network of the mtDNA haplotypes was estimated using TCS ver. 1.2.1 (Clement *et al*. 2000).

To determine if the molecular data for the ingroup taxa included (n = 43) was clock-like a likelihood ratio test was performed in PAUP*b10 (Swofford 2003). Our data does appear to be clock-like (-lnL clock-enforced = 1380.74, -lnL unconstrained tree = 1364.33 χ^2 = 32.82, d.f. = 41, P = 0.81); as a consequence divergence dating performed in BEAST 1.5.5 (Drummond *et al.* 2006; Drummond and Rambaut, 2007) was conducted with the clock enforced. Following Melo *et al*. (2011), we used an average pairwise substitution rate of 4.95% per million years for the mtDNA (mean = 2.475% per lineage, stdev. = 0.0035; 95% HPD $1.899 \times 10^{-2} - 3.051 \times 10^{-2}$) for the estimation of divergence times. This rate is derived from calibration estimates among other *Zosterops* taxa distributed among islands in the Indian and Atlantic Oceans (Warren *et al*. 2006, Melo *et al*. 2011). Confidence can be placed in this estimation as Moyle *et al*. (2009) obtained similar rate estimates for white-eyes distributed in the southwest Pacific based on a different taxon set, independent geological points and different analytical methods (see also Melo *et al*. 2011). BEAST runs we conducted for 5×10^7 generations, with the MCMC chains sampled every 5×10^3 generations; the first 3×10^3 samples were discarded as the burn-in.

4.3.4. Phylogenetic inference – Nuclear DNA and combined analyses

To be able both to infer genealogies and to estimate demographic parameters from nuclear DNA sequence data it is necessary to resolve the bi-allelic phase. All nuclear introns

had more than one polymorphic site (SNP). As a consequence a Bayesian method implemented in the program PHASE (Stephens *et al.* 2001, Stephens and Donnelly 2003) was used to resolve (posterior probability > 0.75) the bi-allelic phase of all linked polymorphisms.

Bayesian and likelihood analyses were performed on the individual data sets separately, as well as on a combined mitochondrial and nuclear data set using MRBAYES version 3.1.2 (Huelsenbeck & Ronquist 2001) and RAXML (Stamatikis 2006), using the same conditions as described above. Individual networks of the phased nuDNA alleles were estimated using TCS ver.1.21 (Clement *et al.* 2000). In addition, a multi-locus nuDNA network was constructed in SPLITSTREE 4.11.3 (Huson and Bryant 2006) from a distance matrix generated using POFAD 1.03 (Joly and Bruneau 2006). As input for POFAD we used uncorrected p-distances for each locus generated using PAUP*b10 (Swofford 2003). For this analysis we only included individuals for which: 1) all four nuclear loci could be satisfactorily phased, and 2) when sequences for all four loci were available.

Individual assignment among the four putative *Zosterops* taxa was determined from the phased nuclear sequence data using STRUCTURE 2.3.3 (Pritchard *et al.* 2000, Hubisz *et al*. 2009). The length of the burn-in was set at 1 x 10^5 repetitions, followed by 1 x 10^6 MCMC repetitions. Individuals are assumed to have mixed ancestry and correlated allele frequencies, therefore the admixture model was used and the LOCPRIOR option implemented. Simulations were conducted for 1 through 6 populations (*K*), and 10 replicates were performed per value of *K* (60 simulations altogether). To ease visual interpretation, output from STRUCTURE was modified using DISTRUCT, version 1.1 (Rosenberg 2004).

4.3.5. Species tree inference

We estimated the species tree for the four southern African morphospecies (Chapter 2) using the approach (STAR-BEAST, Heled and Drummond, 2010) implemented in BEAST

1.5.6 (Drummond *et al.*, 2006; Drummond and Rambaut, 2007). We used a strict molecular clock model for all loci and a HKY + Gamma (four rate categories) model of nucleotide substitution for each data partition. The tree prior was set to the Yule Process with a continuous and constant root. A normal prior distribution (Alpha 3.0, Beta 0.0015) was set for the species-population mean parameter. All other parameters were set to the default. We ran the chains for 5 x 10^7 generations and repeated the analysis three times. TRACER version 1.5 (Rambaut and Drummond, 2007b) was used to help ensure that our sampling of the posterior distribution for all parameters had reached a sufficient effective sample size (ESS) for meaningful parameter estimation. The MCMC chains were sampled every 5 x 10^3 generations and the first 6 x 10^3 samples were discarded as the burn-in. Sampling design for species tree inference is crucial for obtaining tree accuracy, with the addition of multiple loci and several individuals per taxon being considered as important factors to aid resolution of relationships among closely related taxa (McCormack *et al*. 2009). With this is mind, for each of the five loci sequenced, between 9 and 13 individuals per taxon were used for the species tree reconstruction.

4.3.6. Morphospace and environmental analyses

Percentage underpart plumage colour variation was used to place individuals into phenotypic morphospace, since very little taxonomic information is added from standard avian morphometric measurements (i.e. tarsus-, bill-, wing- and tail-length; Chapter 2). Percentage colour scores were estimated from a digital grid superimposed over photographs of individuals taken in the field (see Chapter 2 for further details). Individuals were analysed using individual assignment/clustering methods without being classified *a priori* by taxon. In order to identify patterns of hierarchical similarity, Euclidean distance was chosen as the

similarity coefficient and multi-dimensional scaling analyses were performed on the colour score data, using PRIMER 6.1.5 (Plymouth Marine Laboratory, U.K.).

To determine whether southern African *Zosterops* taxa occupy distinct climate envelopes as suggested by their apparent habitat affinities (Moreau 1957, Chapter 2), environmental data was collected for each of the sampling localities. High resolution (2.5 m) bioclimatic data (www.worldclim.com) was imported into DIVA-GIS, version 7.1.7.2 and the 19 bioclimatic variables were extracted for each of the sampling localities. A multi-dimensional scaling analysis was conducted on the bioclim data to determine potential multivariate grouping between localities. When grouping was observed between localities, a principle component analysis was performed to determine which environmental variables contributed to distinguishing among groups of localities.

4.4. Results

4.4.1. Zosterops *phylogeny – Mitochondrial DNA*

In both the Bayesian and Maximum Likelihood mitochondrial DNA trees (n=73 individuals; Fig. 4.2) three clades are strongly supported. *Zosterops senegalensis* is sister to a clade containing a mix of *Z. capensis* and *Z. virens*, and *Z. pallidus* forms a clade basal to the clades above. Within the *Z. pallidus* clade, four individuals with a *Z. capensis* phenotype are found to be interspersed with pure *Z. pallidus* individuals. All these *Z. capensis* individuals are from Aliwal North (Fig. 4.1). No individuals with a *Z. pallidus* phenotype are interspersed with individuals in the *Z. capensis*/*Z. virens* clade. Within the *Z. capensis*/*Z. virens* clade but with low support (Fig. 4.2), *Z. capensis* individuals from the Western Cape form a subclade.

The mtDNA parsimony network (Fig. 4.3) recovers the same results as for the BI and ML topologies, with *Z. pallidus* haplotypes most divergent, and hence this subnetwork does not connect to the remainder of the network within the 95% statistical parsimony connection

limit. A greater number of mutations are observed among *Z. capensis*, *Z. virens* and *Z. senegalensis* haplotypes than among *Z. pallidus* haplotypes.

4.4.2. Zosterops *phylogeny – Nuclear DNA*

The two autosomal loci (GAPDH and TGFβ2) and one of the sex-linked loci (MUSK) show evidence of a split between *Z. pallidus* alleles and alleles of the other taxa in the statistical parsimony networks (Fig. 4.4). The other taxa (*Z. capensis*, *Z. senegalensis* and *Z. virens*) largely share nuclear alleles, particularly common alleles placed centrally in the network. For the remaining sex-linked locus, CHD1, most individuals share a common allele; however, *Z. senegalensis* individuals have a high proportion of unique alleles.

STRUCTURE provided consistent results over the 10 replicated runs for each value of *K*. The probability of the data (LnPr L(*K*)) was maximum at *K* = 3; however similar probability levels were also attained for *K* = 4 (Fig. 4.5a). Following the methodology of Evanno *et al*. (2005), the highest deltaK (ΔK) value is attained at *K* = 3 (Fig. 4.5b). The three clusters, comprising of 75 individuals, recovered by STRUCTURE (Fig. 4.6) correspond to the well-supported clades estimated in the mtDNA phylogeny: (1) *Z. capensis*/*Z. virens,* (2) *Z. pallidus*, and (3) *Z. senegalensis*. Sampling localities Taba Nchu, Aliwal North, Nova Vita and Tarkastad showed evidence of the presence of hybrid individuals, in agreement with morphology (Chapter 2, and below). As species tree methods are only able to deal with incomplete lineage sorting and not hybridization, to further investigate the true phylogenetic relationship of southern African *Zosterops* taxa, all individuals from sampling localities with evidence of hybridization were removed from subsequent coalescent-based analyses.

4.4.3. Gene trees versus Species trees

In combined mtDNA and nuDNA analyses, 43 individuals were included (86 alleles in the nuDNA after phasing: 26 *Z. pallidus*, 18 *Z. virens*, 22 *Z. capensis*, and 20 *Z. senegalensis*). The multi-locus allele network (Fig. 4.7) provides further support for the topology recovered by the BI and ML analyses of the mtDNA (Fig. 4.8). *Zosterops senegalensis* groups closely to *Z. capensis* and *Z. virens*; there is no evidence of any partitioning within the *Z. capensis*/*Z. virens* clade, and *Z. pallidus* is quite distinct.

The concatenated mtDNA and nuDNA BI and ML trees (Fig. 4.8) recover the same relationships among taxa as depicted in the multi-locus allele network (Fig. 4.7). There is high support for the basal position of *Z. pallidus*, with *Z. senegalensis* being sister to the *Z. capensis*/*Z. virens* group. The species tree estimated using phased allelic data in STAR-BEAST recovers the same set of relationships as the concatenated DNA dataset: the basal position of *Z. pallidus* among southern African *Zosterops* taxa, and the placement of *Z. senegalensis* as sister to *Z. capensis* and *Z. virens* (Fig. 4.9).

4.4.4. Estimation of divergence times

The dating analysis suggests that *Z. pallidus* diverged from other *Zosterops* taxa 771 800 ya (95% CI: 464 700 – 1 111 900 ya) while the split between *Z. senegalensis* and the *Z. capensis*/*Z. virens* clade is estimated to be more recent at 308 100 ya (95% CI: 180 600 – 455 000 ya). The estimated split of *Z. capensis* and *Z. virens* is estimated to occur at 277 500 ya (95% CI: 155 300 – 408 100 ya), although low support values on the species tree (Fig. 4.9) suggests that these two taxa are yet to fully diverge.

4.4.5. Morphospace and environmental analyses

Phenotypically, *Zosterops* fall into four distinct groups (Fig. 4.10a) based on underpart plumage colouration, an important taxonomic character in delineating southern African *Zosterops* taxa. A single *Z. capensis* individual fell within the *Z. pallidus* group, but otherwise all taxa formed distinct groups. This individual was collected from the Kamiesberg region of the Northern Cape of South Africa in the most northerly point of the distribution of *Z. capensis*. Although this individual has the distinct cinnamon flank of *Z. pallidus*, it has a mix of grey- (typical of *Z. capensis*) and white-belly (typical of *Z. pallidus*) and was captured together with other phenotypically pure *Z. capensis* individuals. It is possible that *Z. pallidus* may co-occur with *Z. capensis* at this locality; however, no individuals were captured or recorded there during the sampling effort.

The environmental variables also tend to group collecting localities by putative taxon (Fig. 4.10b). Localities that fall on the boundary between the ranges of *Z. capensis* and *Z. virens* (Grahamstown and Kasouga) fall in the zone between these taxon localities. Localities where both *Z. pallidus* and *Z. capensis* individuals were collected (Aliwal North, Nova Vita and Taba Nchu), fall on the periphery of either of the two groups. However, Aliwal North does appear to be an intermediate locality, not seeming to tend toward either *Z. capensis* or *Z. pallidus* environmental conditions. The first principle component accounted for 42.2% of the variation among localities, separating *Z. senegalensis*, *Z. virens* and coastal *Z. capensis* localities from *Z. pallidus* and inland *Z. capensis* localities. Three main variables accounted for the majority of the variation: annual temperature range (-0.346), temperature seasonality (-0.344) and mean monthly temperature range (-0.338). The second principle component separated *Z. capensis* from the other taxa, with seasonal precipitation (-0.405) accounting for the variation. As is expected, the environmental variables for the *Z. senegalensis* localities (Nyika and Zomba in Malawi) group away from the South African localities.

4.5. Discussion

4.5.1. Molecular taxonomy of southern African Zosterops

Southern African *Zosterops* taxonomy has never been fully resolved, with contrasting views on the relationship and placement of the four main colour forms that occur within the region (i.e. Gill, 1936, Roberts 1942, Moreau 1957, Clancey 1967, Skead 1967, Hockey *et al.* 2005, van Balen 2008, Chapter 2). In particular, contention has always centred on the relationship between the Grey Cape White-eye *Z. capensis*, the Orange River White-eye *Z. pallidus* and the Green Cape White-eye *Z. virens*. These three taxa have either been lumped, due to the presence of hybrids among forms, as a single taxon (Clancey 1967, van Balen 2008), or split into two separate species (with variable numbers of subspecies) with some permittance of hybridization among delineated taxa (Moreau 1957, Skead 1967, Hockey *et al.* 2005).

The African Yellow White-eye *Zosterops senegalensis* has always been considered sister to these predominantly southern African distributed groups. This phylogenetic scenario has never been questioned, probably due to a lack of geographic overlap among *Z. senegalensis* and the other regional taxa, and due to the lack of putative hybrids between *Z. senegalensis* and the other *Zosterops* taxa of the region. Thus, the placement of *Z. pallidus* basally to the other *Zosterops* taxa in the molecular analyses is an unexpected result.

4.5.2. Is there agreement with other lines of evidence?

A morphological reassessment of southern African *Zosterops* systematic relationships (Chapter 2) revealed four phenotypically discernable taxa. In contrast, analyses of songs and contact calls (Chapter 3) were only able to distinguish *Z. pallidus* from other *Zosterops* taxa. The songs of *Z. capensis*, *Z. senegalensis* and *Z. virens* were indistinguishable. While not fully congruent with either the morphological or the vocalization results, the molecular

phylogeny presented here shows similarities to both. *Zosterops pallidus* is highly divergent from the other *Zosterops* taxa, which corresponds to the differences observed both phenotypically and vocally. The placement of *Z. senegalensis* as sister to the clade consisting of *Z. capensis* and *Z. virens* is surprising; however, song character analyses (Chapter 3) did point towards the three taxa being closely related. Although these closely related taxa have diverged fairly recently (~ 300 000 ya), differences in various characters have evolved at different rates.

Plumage colouration is thought to be a trait that can evolve relatively quickly, particularly in *Zosterops* (Milá *et al.* 2010), and thus the morphological differences observed here are to some extent not unexpected. At present, the genetic basis of the plumage polymorphism is unknown. Divergence in vocalizations can be influenced by habitat type (i.e. Morton 1975) where contrasting adaptive pressures can drive the production of dissimilar song and call types. The similarity in structure of habitat (dense scrub to forest) occupied by *Z. capensis*, *Z. senegalensis* and *Z. virens* is likely to account for the similarity of song traits, even though *Z. senegalensis* is divergent from the other taxa at a molecular level. Alternatively, with a short divergence time extimate presented here, songs of these taxa may have yet had time to diverge. The contrasting habitat type of *Z. pallidus* (riverine corridor, semi-desert scrub) together with the age of divergence (~ 770 000 ya) from the other regional *Zosterops* taxa has allowed for the evolution of the divergent vocalization characteristics found in this taxon by either selection or cultural drift.

4.5.3. Non-sister taxon hybridization

Although all the genetic data presented here suggests that *Z. pallidus* and *Z. capensis* are not sister taxa, and that they shared a common ancestor ~ 770 000 ya, strong evidence for hybridization between the two taxa is shown in the independent mtDNA data (Figs. 4.2 &

4.3) and combined nuDNA data datasets (Fig. 4.6), as well as in phenotype (Chapter 2) and in vocalizations (Chapter 3). The intermediate environmental conditions, and in particular temperature and rainfall, of localities (Aliwal North, Nova Vita, Taba Nchu) of overlap between *Z. pallidus* and *Z. capensis* are likely to have facilitated secondary contact, and subsequent interbreeding, between these taxa. Further tests (i.e. Peters *et al*. 2007) may be needed to rule out incomplete lineage sorting as a possibility for the sharing of pattern observed here. Hybridization is not expected to be restricted to these two taxa as morphological intergradation has also been documented between *Z. pallidus* and *Z. virens* (Moreau 1957, Clancey 1967, Chapter 2). No evidence of molecular hybridization between these two taxa was found in this study.

4.5.4. African white-eye diversification

Much attention has been given to the initial radiation of the Zosteropidae in Asia, the Pacific, and on the islands of the Indian Ocean (Warren *et al*. 2006, Moyle *et al*. 2009), which harbour a tremendous amount of white-eye diversity. Most of this diversity is a result of the adaptability and colonizing ability of Zosteropidae, with many endemic species being found on islands of South East Asia and the Indian Ocean (Mees 1953, Mayr 1965, Warren *et al*. 2006). Colonization of Africa by white-eyes is likely to have occurred via a single event through the north of the continent (Warren *et al*. 2006), with subsequent colonization of the Gulf of Guinea (including Mount Cameroon) and Madagascar (and surrounding islands) occurring around 1.25 and 1.2 mya, respectively (Melo *et al*. 2011). Each of these island systems shows high levels of white-eye diversity, particularly at the subspecies level. For a continental region, southern Africa displays fairly high white-eye diversity, which is partly due to the environmental variability of southern Africa coupled with rapid morphological change typical of many *Zosterops* taxa (Clegg *et al*. 2002, 2008, Philimore *et al*. 2008, Milá

et al. 2010). The taxa of the southern African subregion shared a common ancestor ~ 770 000 ya, with the ancestor of *Z. pallidus* either dispersing south after being separated from the other taxa by an arid corridor that ran from east Africa to southwestern Africa (van Zinderen Bakker 1969) and then adapting to the arid environments it currently occupies, or by adapting to the conditions of the arid corridor before dispersing south into southwestern Africa. Many species of both plants and mammals have followed this colonization route in southern Africa, with taxa found in the arid regions of the Horn of Africa and the Namib Desert being closely related (Honeycutt *et al*. 1987, Jürgens 1997, Herron *et al*. 2005, Smit *et al*. 2011). A more detailed understanding of the colonizing route of *Z. pallidus* will be achieved with the addition of further samples of *Zosterops* taxa from East Africa.

The southern dispersal route of the other white-eye taxa is likely to have occurred down the east coast of Africa, although a scenario of continental recolonization from Madagascar cannot be ruled out (Warren *et al*. 2006). Presently, *Z. senegalensis* and *Z. virens* are ecologically separated, occurring in the deciduous bush of the low-veld and the evergreen forests of coastal acacia, respectively (Moreau 1957). The exploitation of the novel evergreen forest habitat by the *Z. capensis*/*Z. virens* ancestor approximately 300 000 ya is likely to have led to the divergence from *Z. senegalensis*.

Even with pronounced differences in plumage colouration, the molecular data, in agreement with vocalization data (Chapter 3), does not provide support for the classification of *Z. capensis* and *Z. virens* as discrete species. The molecular evidence presented here does however, point to independent evolutionary trajectories within the *Z. capensis*/*Z. virens* clade. Evidence of speciation within habitats has been reported in threespine stickelback fishes (McKinnon *et al.* 2004, Kume *et al.* 2010), and may be the case for these *Zosterops* taxa. The green-bellied *Z. virens* is found to occur in evergreen forested habitats (Moreau 1957, Hockey *et al*. 2005), whereas the grey-bellied *Z. capensis* occupies the Fynbos biome.

Several Fynbos endemic birds are found in South Africa (Orange Breasted Sunbird *Anthobaphes violacea*, Protea Seed-eater *Serinus leucopterus*, Cape Sugarbird *Promerops cafer*, Cape Rock-jumper *Chaetops frenatus*; Hockey *et al*. 2005): other Fynbos restricted taxa include Dwarf Chameleons *Bradypodion* spp. (Tolley *et al*. 2006) and the Cape Rock Elephant Shrew *Elephantulus edwardii* (Smit *et al.* 2007). Barriers to dispersal in the northwest appear to occur at the Knersvlakte mountain range (Matthee and Robinson 1996, Matthee and Flemming 2002, Daniels *et al*. 2010, Portik *et al*. 2011), while restrictions to the east can be attributed to climate (particularly a switch from winter to summer rainfall). While the possible climatic barrier to dispersal appears to restrict dispersal of *Z. capensis* towards the east, northern dispersal is not being restricted by the Knerslvlakte mountain formation.

The morphological diversification of southern African *Zosterops* taxa appears to have occurred fairly recently, and likely to be driven by the colonization of novel environmental 'islands'. Diversification in island *Zosterops* taxa often occurs with a reduction in gene flow (Milá *et al*. 2010). Thus, further work investigating the extent of gene flow between distinct morphospecies (Chapter 5) will help determine the balance between gene flow and adaptation in promoting the observed phenotypic diversity.

The results of this chapter also indicate that using interbreeding between taxa as a criterion for determining species relationships can be misleading. Given the placement of *Z. pallidus* as ancestral to *Z. senegalensis*, and *Z. senegalensis* being sister to a clade comprising both the Grey and Green Cape White-eye (*Z. capensis* and Z. *virens*), secondary contact and a lack of the formation of adequate pre-zygotic and post-zygotic barriers is predicted to have allowed interbreeding between *Z. pallidus* and *Z. capensis* and, potentially, *Z. virens* to take place. No evidence of interbreeding of *Z. senegalensis* with the other taxa is observed, suggesting reproductive isolation of this taxon. Interestingly, there is interbreeding between *Z. capensis* and *Z. virens* (Moreau 1957, Skead 1967). Even though phenotypic hybrids are

known to occur (Chapter 2), the low power of the nuclear markers to distinguish between taxa means that no evidence of hybrid individuals could be detected.

Other phylogeographic studies for vertebrate taxa within the region (Tolley *et al*. 2006, Smit *et al*. 2007, Swart *et al*. 2009) have focused primarily on mtDNA and generally revealed little or no mixing of mtDNA lineages. However, in the Karoo Scrub-Robin mtDNA divergence has been shown to have occurred as a consequence of selection with considerable nuDNA gene flow taking place across the species range (Ribeiro *et al*. 2011). Similar disparities may exist for other taxa and a multi-locus approach is strongly advocated to be adopted when investing phylogeographic structure across heterogeneous landscapes such as in southern Africa.

Tables

Table 4.1: The number of *Zosterops* individuals used per sampling locality. Taxa were classified *a priori* from phenotypic characters (underpart colouration and morphometric measurements). Sample numbers correspond to voucher specimens (* = digital vouchers, † = no voucher) housed in the Museum of Vertebrate Zoology and Field Museum.

Phenotypic classification	Locality	N	Sample name
Z. capensis	Aguhlas National Park (ANP)	2	JF1956*, JF1929
Z. capensis	Aliwal North (AN)	4	GO102, GO107, GO116, GO153,
Z. capensis	Anysberg National Park (ANY)	2	JF2681*, JF2683
Z. capensis	Beaufort West (BW)	4	JF2143†, JF2145†, JF2166†, JF 2173†
Z. capensis	Bontebok National Park (BNP)	2	JF1999, JF2004*
Z. capensis	Cape St. Francis (CSF)	1	JF500*
Z. capensis	De Rust (DR)	2	AP01301*, AP01310*
Z. capensis	Grahamstown (GHT)	1	JF557*
Z. capensis	Kamiesberg (KMB)	3	GO30, AP01038*, AP01049*
Z. capensis	Nova Vita (NV)	2	GO269, RB727
Z. capensis	Picketberg (PKB)	2	RSA157, WE0018†
Z. capensis	Taba Nchu (TN)	3	RSA213, RSA216, RSA220
Z. capensis	Tarkastad (TKS)	5	JF810*, JF815*, JF817*, JF821*, JF825*
Z. pallidus	Aliwal North (AN)	2	GO129, GO152
Z. pallidus	Kimberley (KIM)	5	GAV3001, GAV3004, GAV3008, GAV3009, GAV3018
Z. pallidus	Kuruman (KUR)	2	JF2377, JF2379
Z. pallidus	Nova Vita (NV)	1	GO270
Z. pallidus	Ottoshoop (OH)	1	GO205
Z. pallidus	Sandveld Nature Reserve (SNR)	3	GO181, GO184, GO195
Z. pallidus	Taba Nchu (TN)	2	RSA214, RSA215
Z. pallidus	Vanderbijlpark (VBP)	1	L22008*
Z. pallidus	Vioolsdrift (VD)	5	GO35, HS100†, HS138†, HS168*, HS171*
Z. senegalensis	Nyika (NKA)	6	RB3114 - RB3119
Z. senegalensis	Zomba Plateau (ZOM)	4	GA94343†, JP499, JP500, JP501
Z. virens	Eshowe (ESH)	1	RSA101

Z. virens	Kasouga (KSG)	2	JF564*, JF566*
Z. virens	Louis Trichard (LT)	2	GO227, GO240
Z. virens	Melmoth (MEL)	1	RSA124
Z. virens	Morgan Bay (MB)	3	JF746*, JF755*, JF766*
Z. virens	Ngulube Reserve (NGU)	1	GO244

Table 4.2: A list of the primers used for the amplification of the loci used.

Locus	Primers	Reference
ATP6	A8PWL: CCTGAACCTGACCATGAAC CO3HMH: CACATAGTRGACCCCAGCCCATG	Eberhard and Bermingham (2004)
MUSK	I3F: CTTCCATGCACTACAATGGGAAA I3R: CTCTGAACATTGTGGATCCTCAA	Kimball *et al*. (2009)
CHD1	P14: ACTTTTCCAATATGGATGAAGA P9: TAAGGTCTGTCTCAGAYTTRTCNAC	Griffiths *et al*. (1998)
	ZostF: TTATACTACACTGAATATCAGAGCCAC ZostR: ACCGTCAATTCCCATTTCAGG	this study
GAPDH	L890: ACCTTTAATGCGGGTGCTGGCATTGC H950: CATCAAGTCCACAACACGGTTGCTGTA	Friesen *et al*. (1997)
TGFβ2	TGF5: GAAGCGTGCTCTAGATGCTG TGF6: AGGCAGCAATTATCCTGCAC	Primmer *et al*. (2002)

Figure legends

Figure 4.1: Biome map showing the sampling localities of samples used to determine the taxonomic relationship within southern African *Zosterops* taxa. Blue dots = *Z. capensis* localities, red dots = *Z. pallidus* localities, green dots = *Z. virens* localities, yellow dots = *Z. senegalensis* localities, black dots = localities where multiple taxa were collected. Full names of abbreviated locality names are shown in Table 4.1.

Figure 4.2: Phylogeny of southern African *Zosterops* based on ATP6 DNA sequences (n = 73) using a partitioned Bayesian analysis and a partitioned Likelihood analysis. Two Asian outgroup taxa were used (Ranongga White-eye *Zosterops splendidus* and Chestnut-flanked White-eye *Z. erythropleurus*). Values above branches are posterior probabilities, whereas those below are bootstrap support values.

Figure 4.3: A parsimony ATP6 haplotype network (n = 73) of the four southern African *Zosterops* taxa. Unconnected haplotype networks are separated by a 95% connection limit. Blue = *Z. capensis*, Orange = *Z. pallidus*, Green = *Z. virens* and Yellow = *Z. senegalensis*. Circles are proportional to the number of haplotypes they contain and open circles represent unsampled haplotypes.

Figure 4.4: Parsimony allele networks of phased GAPDH, TGFβ2, CHD1 and, MUSK of the four southern African *Zosterops* taxa. Blue = *Z. capensis*, Orange = *Z. pallidus*, Green = *Z. virens* and Yellow = *Z. senegalensis*. Circles are proportional to the number of haplotypes they contain and open circles represent unsampled haplotypes.

Figure 4.5: The number of populations estimated from STRUCTURE: a) The mean estimate of the probabilities of the data (LnPr L(*K*)) over 10 replicated runs for each value of *K*, b) Values of Δ*K* (calculated as $\Delta K = m|L''(K)|/s[L(K)]$; Evanno *et al*. 2005) plotted against values of *K*.

Figure 4.6: Structure plot based on the combined nuclear data set (autosomal: GAPDH and TGFβ2, and sex-linked: CHD1 and MUSK) of 75 southern African *Zosterops* individuals. The groupings of the four phenotypic *Zosterops* taxa are shown (*Z. pallidus, Z. capensis, Z. virens* and *Z. senegalensis*). Each individual is represented by horizontal lines divided into three coloured segments that represent membership to each cluster. Localities where multiple

taxa where collected are represented: Taba Nchu, Aliwal North, Nova Vita and Tarkastad, along with the taxa found at these localities.

Figure 4.7: A combined non-standardized multi-locus nuDNA (autosomal: GAPDH, TGFβ2 and sex-linked: CHD1, MUSK) network.

Figure 4.8: Phylogeny of southern African *Zosterops* taxa based on a combined mtDNA (ATP6) and nuDNA (autosomal: GAPDH, TGFβ2 and sex-linked: CHD1, MUSK) dataset (n = 43) using Bayesian and Likelihood analyses. Values above branches are posterior probabilities, whereas those below are bootstrap support values.

Figure 4.9: A species tree showing the relationship of southern African *Zosterops* taxa. Values above branches are posterior probabilities.

Figure 4.10: A multi-dimensional scaling analysis of: a) the underpart percentage colour scores (grey, green, yellow, cinnamon and white) of the four southern African *Zosterops* taxa. V = *Z. virens*, C = *Z. capensis*, P = *Z. pallidus*, S = *Z. senegalensis*; and b) 19 environmental bioclimatic variables for each of the *Zosterops* collecting localities. Green = *Z. virens*, blue = *Z. capensis*, red = *Z. pallidus*, yellow = *Z. senegalensis*, black = localities with multiple taxa.

Fig. 4.1

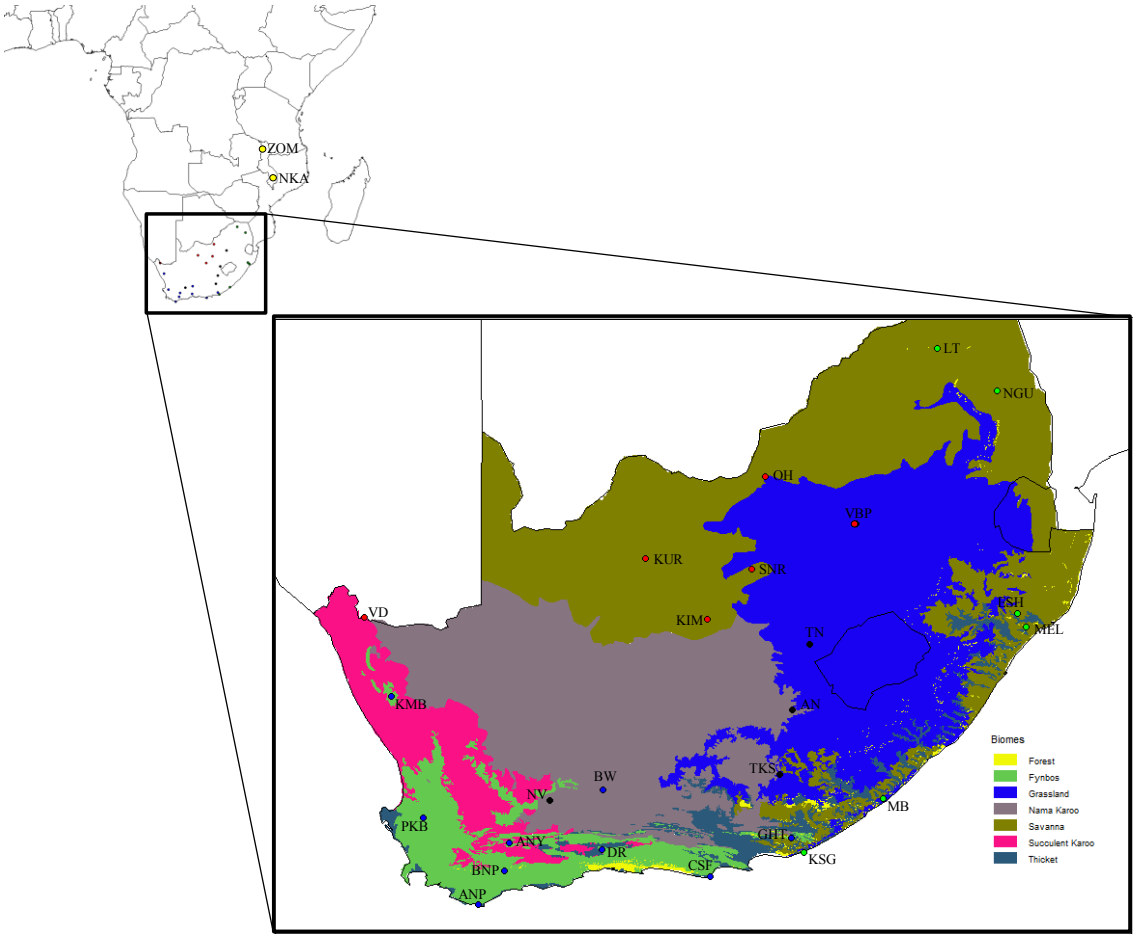


Fig. 4.2

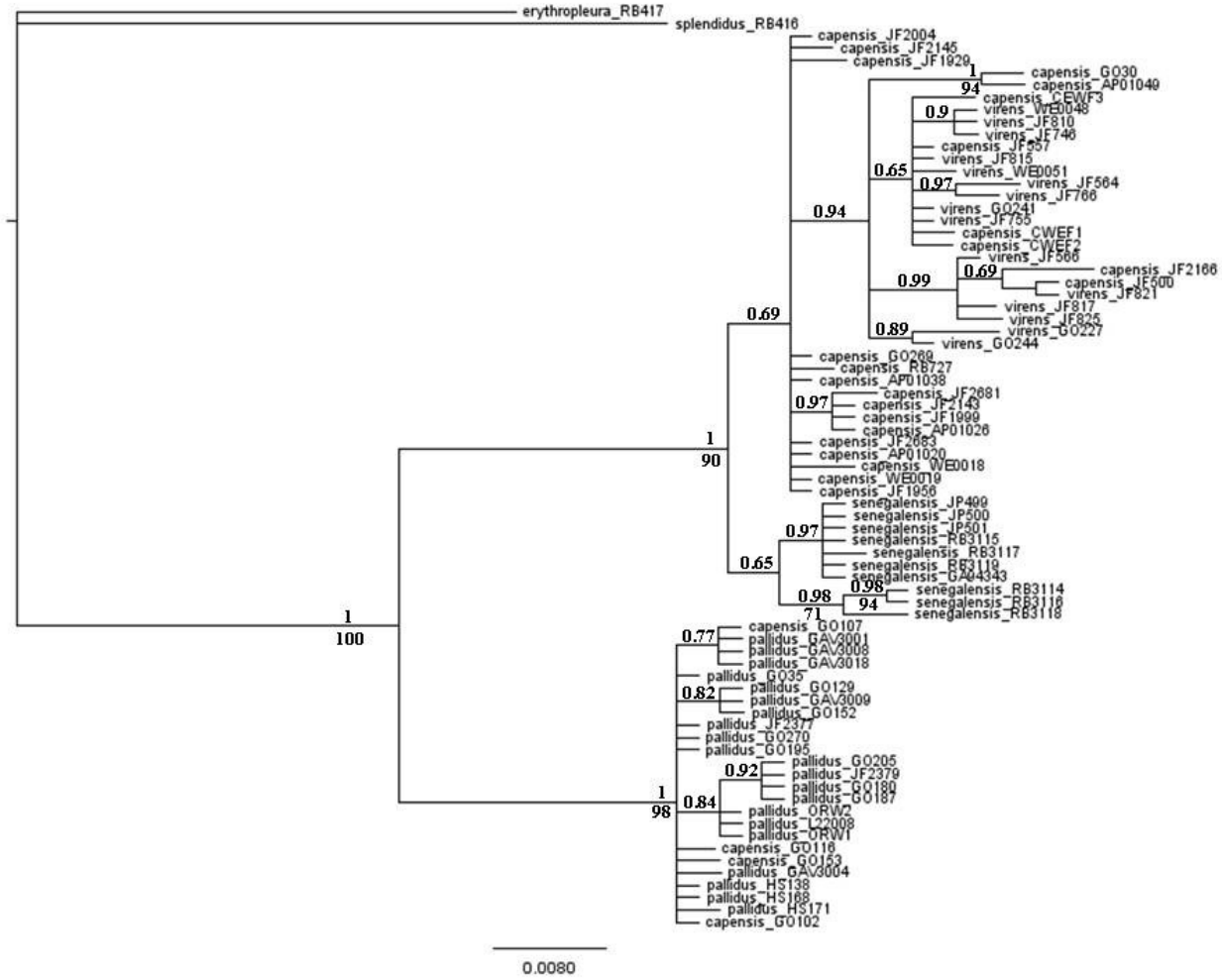


Fig. 4.3

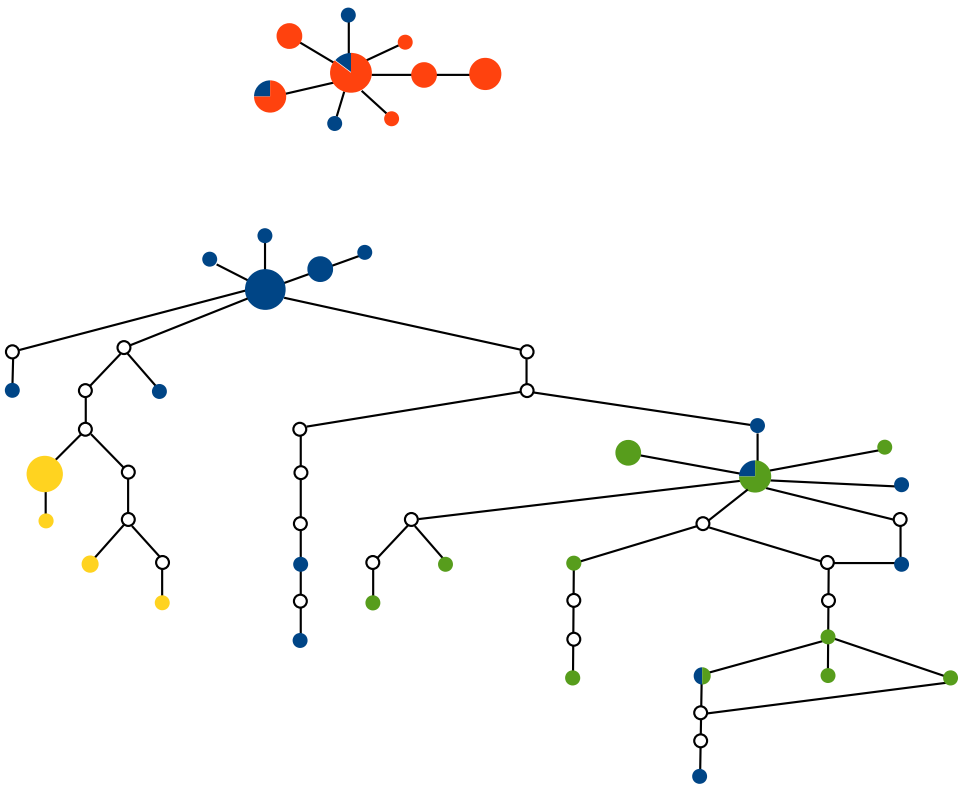


Fig. 4.4

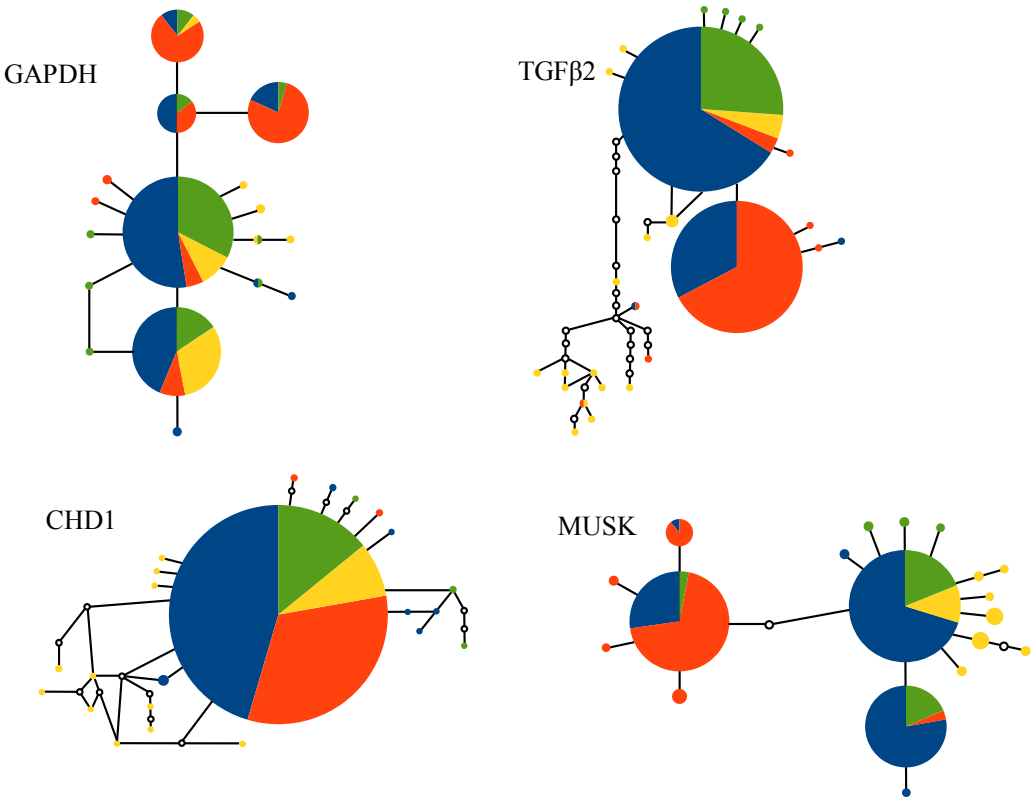


Fig. 4.5a

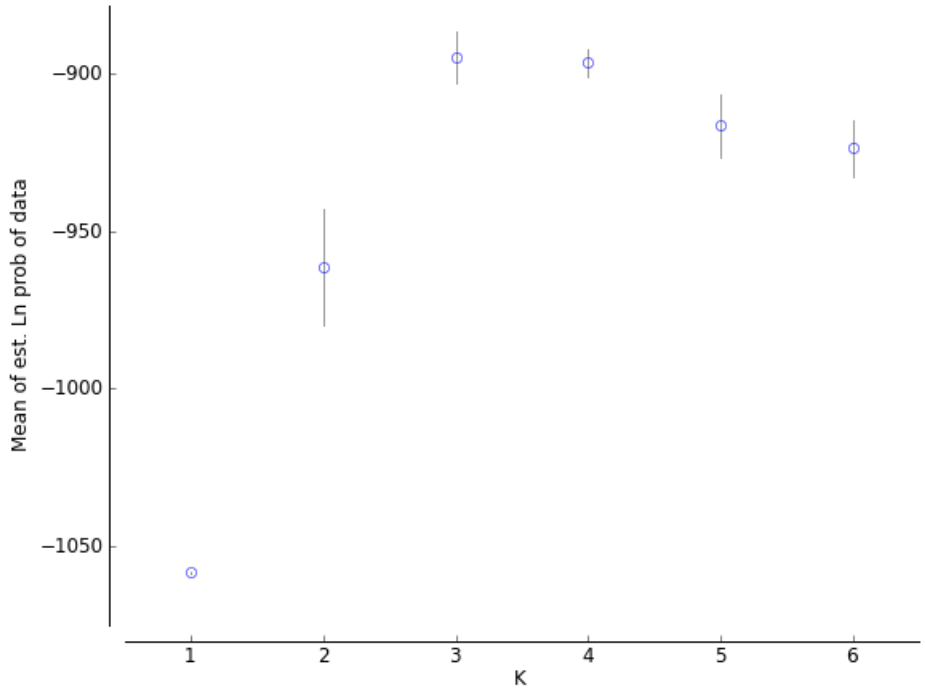


Fig. 4.5b

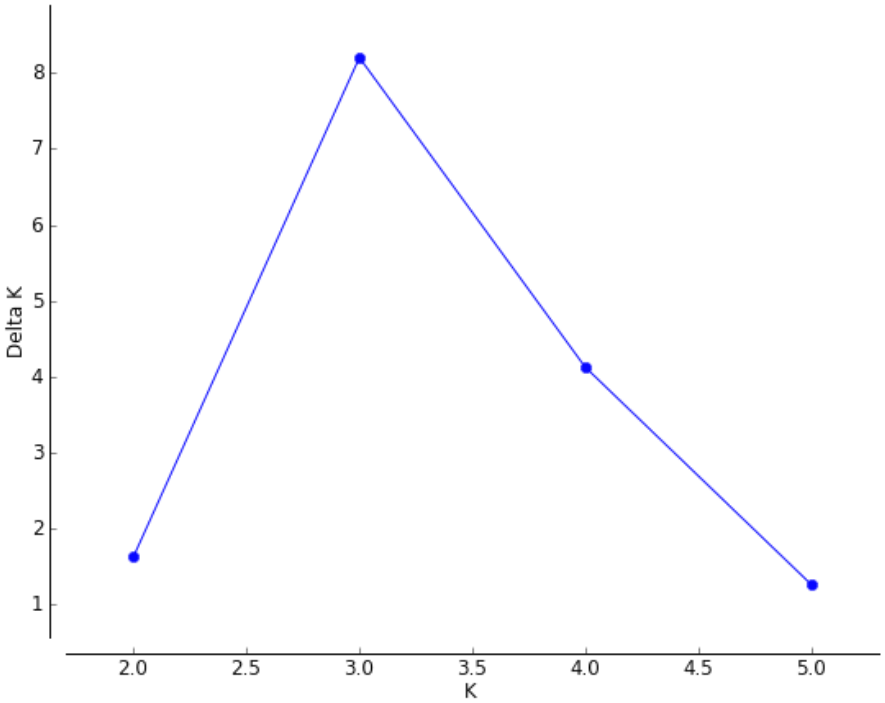


Fig. 4.6

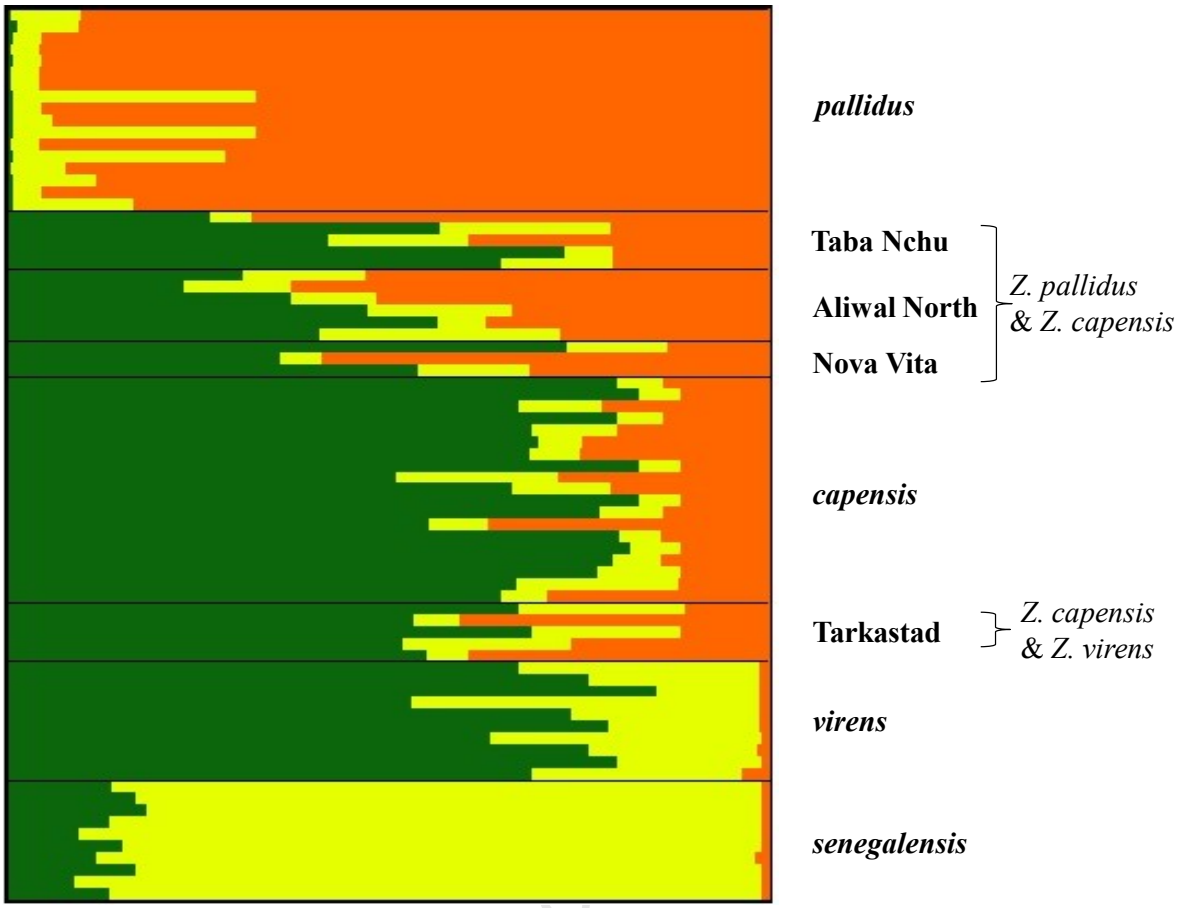


Fig. 4.7

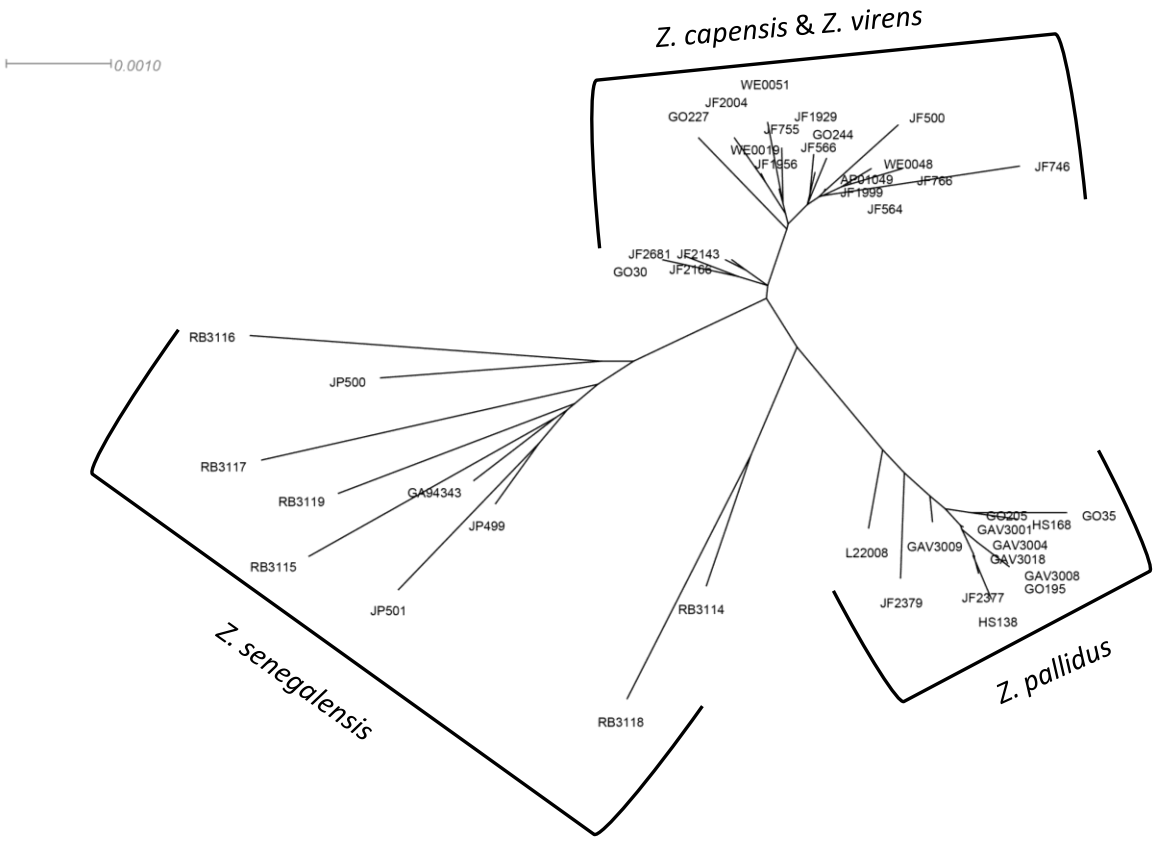


Fig. 4.8

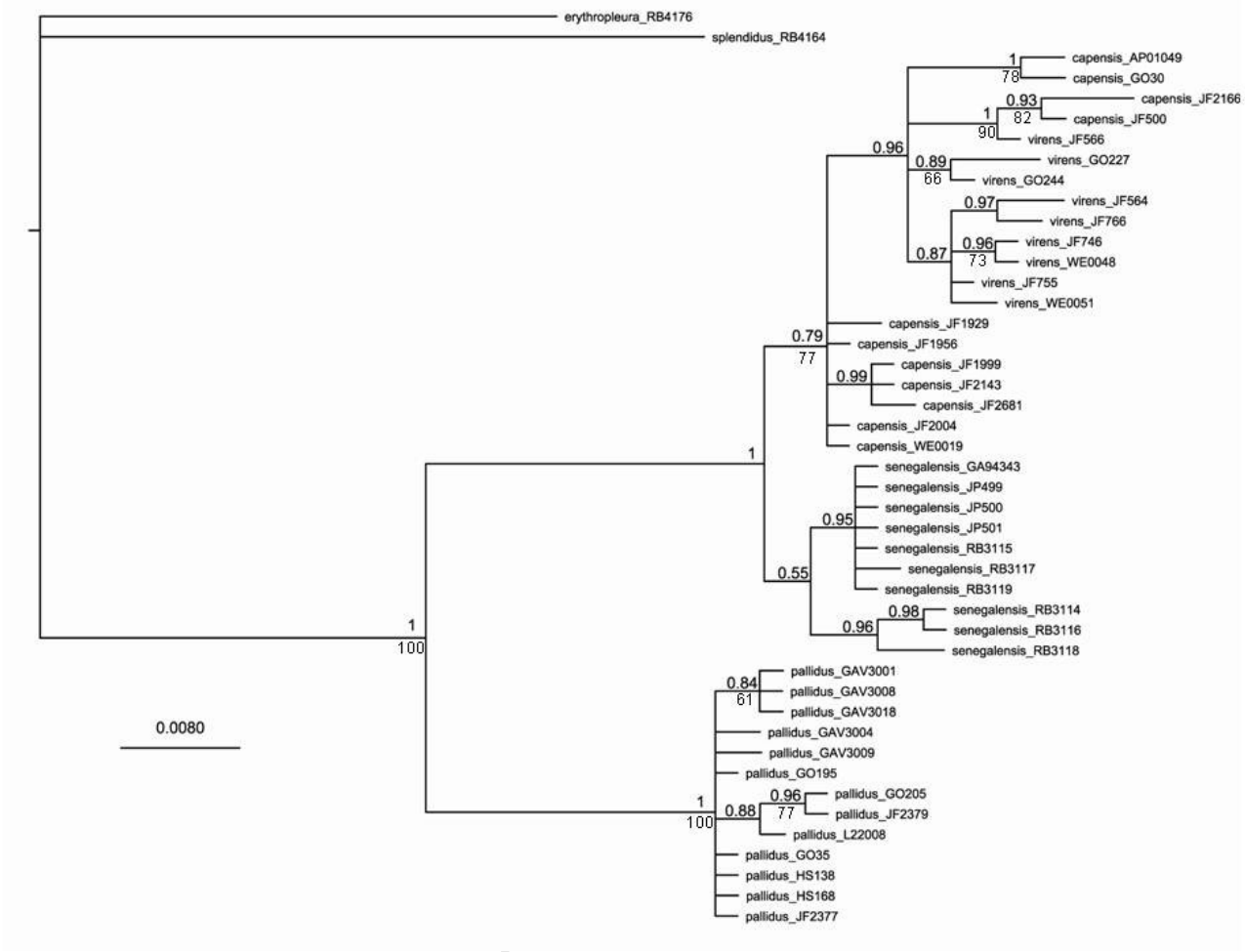


Fig. 4.9

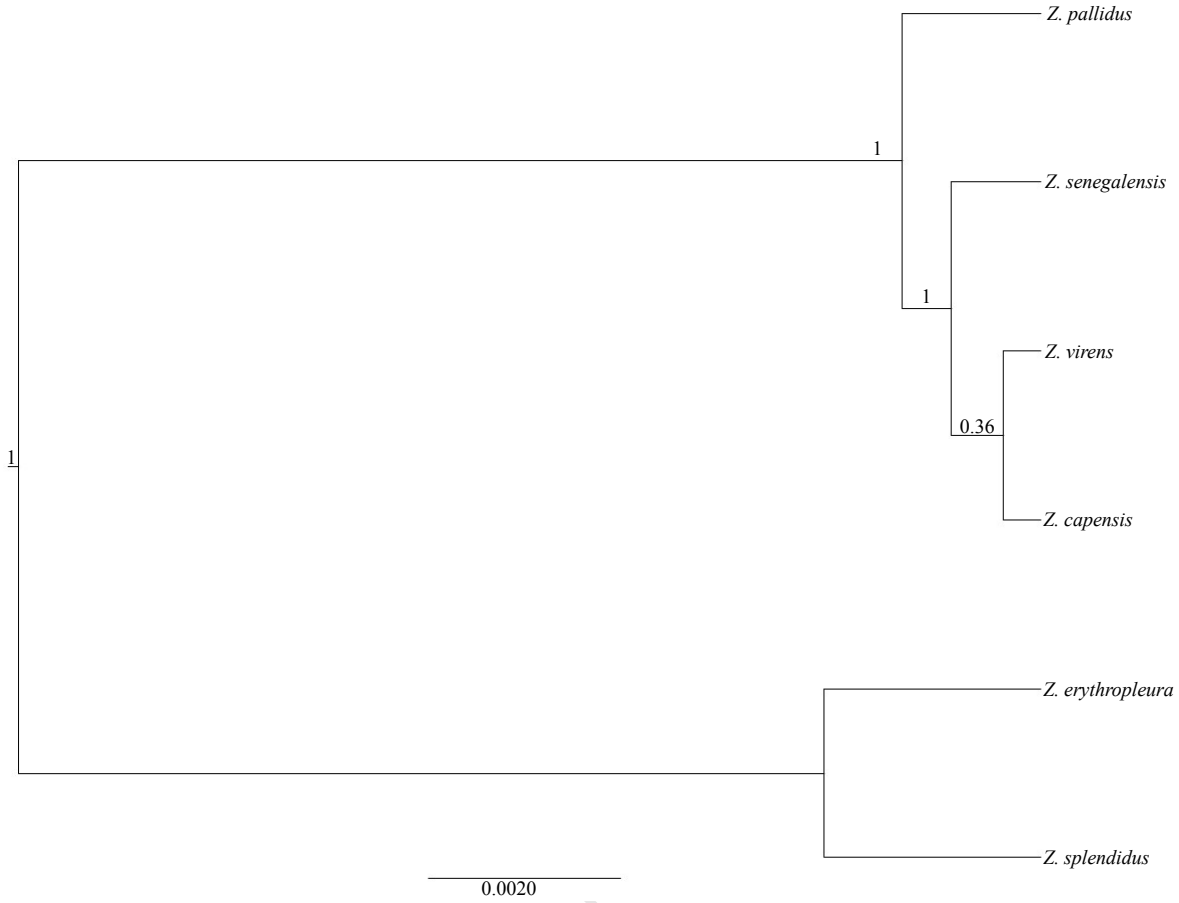


Fig. 4.10a

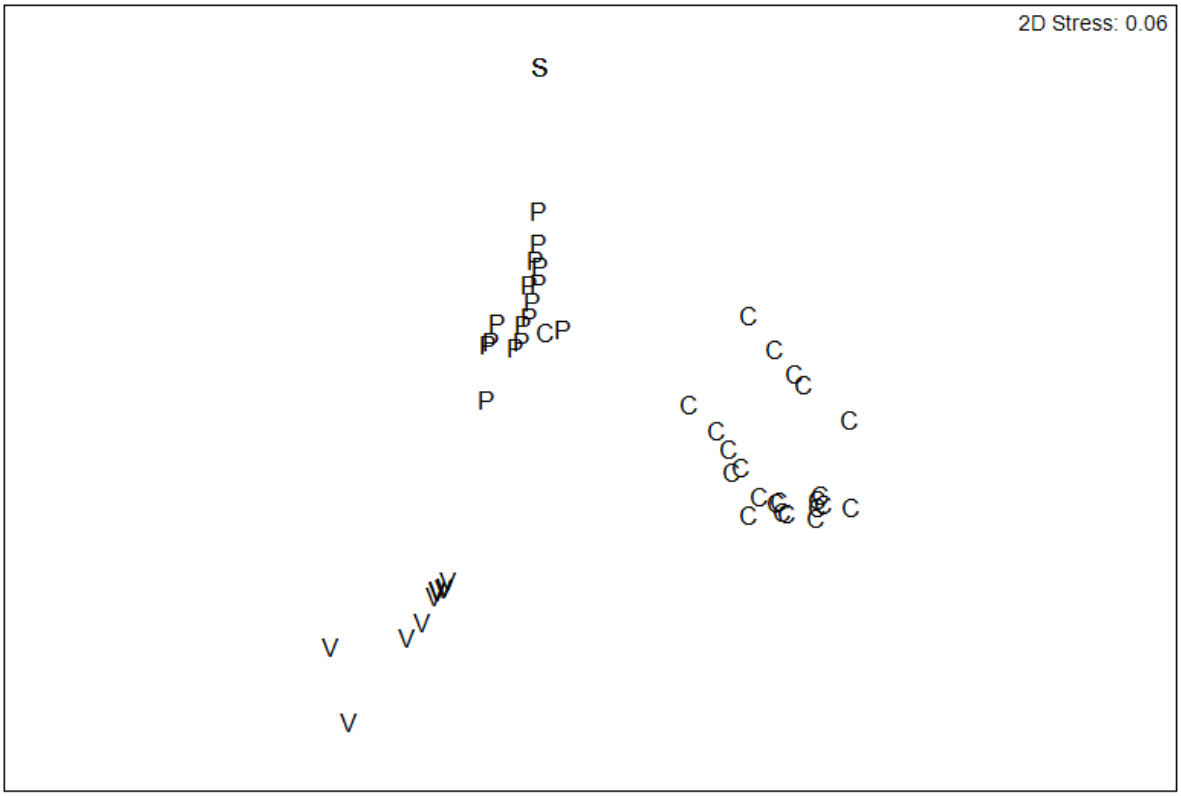
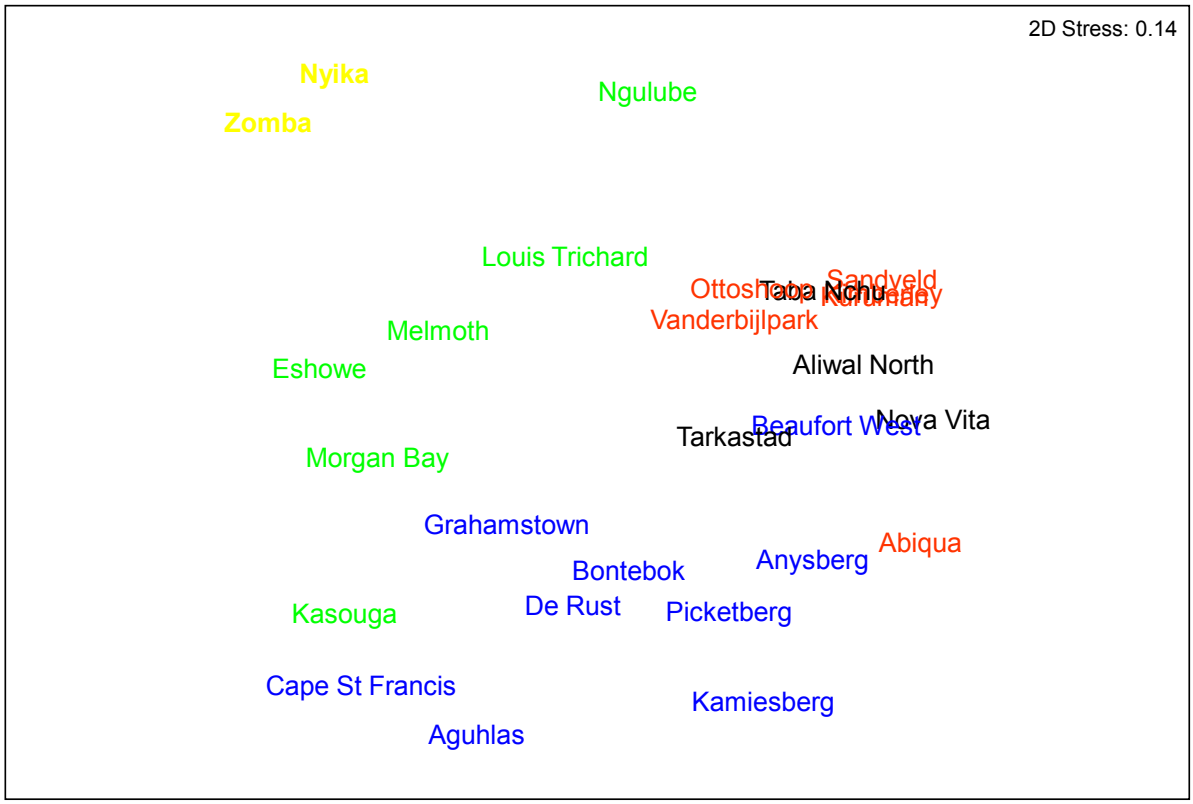


Fig. 4.10b



Chapter 5

Multi-locus phylogeography of southern African White-eyes (*Zosterops* spp.) reveals phenotypic divergence across habitat boundaries with gene flow and introgressive hybridization after secondary contact

5.1. Abstract

While phylogeographic paradigms have been established for much of the Nearctic and Palearctic biogeographical regions, similar paradigms have yet to be established for the entire southern African region. This is in part due to lower levels of research in the region and to the focus on predominantly southwesterly distributed taxa. Using molecular data from four *Zosterops* taxa that together have distributions that cover most of the environmentally diverse biomes found in southern Africa, and by applying phylogeographic, and in particular coalescent analyses, an attempt is made to understand the factors driving current *Zosterops* diversity. The genetic structure of southern African *Zosterops* has been minimally affected by the diverse Biomes and environmental conditions in the region, and the structure is likely due to the historical divergence of these taxa. A Bayesian structure analysis, and to a lesser extent separate gene networks, indicate that *Z. pallidus* and *Z. senegalensis* are separate taxonomic entities, and that introgressive hybridization is occurring between *Z. pallidus* and both *Z. capensis* and *Z. virens*. Coalescent analyses reveal moderate levels of gene flow between these taxa, with IMA results indicating strong asymmetrical migration rates from *Z. pallidus* into *Z. virens*; similar migration rates are expected between *Z. pallidus* and *Z. capensis*. Estimated divergence times for southern African white-eyes are shown to be fairly recent and in conflict with previous, more robust dating analyses, highlighting the affects of recent introgression in obtaining accurate timing estimates from IM and IMA. Even so, the high plumage polymorphism exhibited by *Z. capensis* and *Z. virens* is expected to have arisen recently in spite of high levels of gene flow between these taxa. Adaptation to the novel fynbos biome and associated climatic conditions is suspected to be the driver behind *Z. capensis* plumage colouration, while population expansion associated with habitat expansion during cycles of alternating warm, wet and cool, dry periods during the past 600 000 years is likely to have allowed for secondary contact with *Z. pallidus*.

5.2. Introduction

Phylogeography is a combination of population genetics, phylogenetics and biogeography, and is the biological discipline that uses information from the genetic lineages of closely related taxa (species or subspecies) to understand their contemporary distribution (Avise *et al.* 1987). Phylogeographic research began in earnest in the late 1970s and 1980s (i.e. Avise *et al.* 1979, Lansman *et al.* 1983, Avise *et al.* 1986), and the theory that has developed over the ensuing years has helped form an understanding of the spatial and temporal evolutionary processes shaping current spatial patterns of biodiversity (Avise *et al.* 1987, Avise 2000). Further development in the field (Avise 1998, Beheregaray 2008), particularly the increasing use of molecular technology, better computational power and refined statistical methodologies (see Knowles 2004, Excoffier and Heckel 2006, Knowles 2009a) are all enabling a better understanding of phylogeographic patterns and processes to be formed.

To date, phylogeographic studies, particularly of birds, have predominantly focused on Northern Hemisphere taxa, and in particular on taxa in the Nearctic and Palearctic biogeographical regions (Beheregaray 2008). Research conducted on terrestrial taxa in both these regions has led to the development of evolutionary paradigms of phylogeographic history during the Plio-Pleistocence, particularly the inference and identification of glacial refugia that harboured most of the genetic diversity during this period (Hewitt 2000, 2001, Soltis *et al*. 2006) as well as highlighting patterns of post-glacial range expansion (Taberlet *et al*. 1998, Hewitt 1999).

There have been far less phylogeographic research in southern Africa than in many other biogeographical areas (Beheregaray 2008) and as such, evolutionary paradigms for the region are, at best, speculative. Research conducted further north focusing on Central, Western and Eastern African taxa has shown that the alternating contraction and expansion of

Savanna and Forest biomes has affected the historical distribution and hence current patterns of genetic structure in arid-adapted (e.g. Muwanika *et al.* 2003, Lorenzen *et al.* 2010, Fuchs *et al*. 2011) and forest-adapted (e.g. Evans *et al.* 2004, Anthony *et al.* 2007, Mouline *et al.* 2008, Blackburn and Measey 2009, Bryja *et al.* 2010) vertebrate taxa. Studies of bird phylogeography in the region have hypothesised that either lowland forest (Bowie *et al.* 2004, Ngumbock *et al.* 2009, Marks 2010) or 'sky-island' (Bowie *et al.* 2006, Voelker *et al.* 2010) refugia could be the driving force behind the biodiversity patterns observed. Indeed, as early as the 1960s, Hall (1963), Moreau (1966) and especially, Crowe (1978), Diamond and Hamilton (1980), Crowe and Crowe (1982) and Mayr and O'Hara (1986) speculated on and demonstrated the effects of refugia on speciation in Africa's Forest, Savanna and arid biomes.

Phylogeographic research on southern African vertebrates has predominantly focused on taxa in the southwestern part of the region, particularly on the effects of mountain refugia (e.g. Cape Rock Elephant Shrew Smit *et al.* 2007, 2010, dwarf mountain toads Tolley *et al.* 2010) or habitat fragmentation (e.g. dwarf chameleons Tolley *et al.* 2006, Tolley *et al.* 2008; Angulate Tortoise *Chersina agulata* Daniels *et al.* 2007, Chacma baboons Sithaldeen *et al.* 2009, Southern Rock Agama Matthee and Flemming 2002, Swart *et al.* 2009, Namaqua Rock Mouse Russo *et al.* 2010, skinks Portik 2011) on taxon diversity and radiation. Given this growing body of research it is surprising that almost no phylogeographic studies of birds within the subregion have been published to date. Potentially informative studies have been those on larks (Ryan and Bloomer 1997, 1999; Ryan *et al*. 1998), which revealed deep mtDNA divergence among taxa and across biome boundaries, as well as showing within taxon plumage adaptation that matched the colour of the substrate (Ryan and Bloomer 1997, Ryan *et al*. 1998). More recently, a study of the Karoo Scrub-Robin (Ribeiro *et al*. 2011) recovered west coast and interior mtDNA clades, a result strongly discordant from panmixia inferred from the nuDNA markers, possibly as a consequence of strong selection acting on

the mtDNA genome in this species. Further, no results have been published for vertebrate taxa distributed throughout southern Africa, and hence to date it has not been possible to understand the historical processes that have affected past and current taxon distributions across the diverse biomes of the southern African subregion.

The range of vegetation diversity within southern Africa is extensive, with seven distinct biomes having been described (Fig. 5.1; Cowling *et al*. 2004, Mucina and Rutherford 2006). The most widespread biomes are the Savanna, Nama-Karoo and Grassland habitats (Cowling *et al*. 2004), with the most restricted being the Forest biome, making up less than 0.5% of South Africa's total land area. The vegetation diversity of the region is largely affected by rainfall and topographical variation; South Africa experiences a seasonal west to east precipitation gradient and has an elevated central plateau separated from the coastal region by a mountainous escarpment.

The southern African *Zosterops* taxa provide an opportunity to understand past evolutionary processes that could have driven current biodiversity patterns observed in the region. Four morphologically distinct *Zosterops* taxa (Chapter 2) are found throughout southern Africa (with the exception of Botswana). Each putative taxon falls within distinct climatic and vegetative zones, with areas of overlap tending to occur at boundaries between these zones (Fig. 5.1). Typical *Z. capensis* birds (grey-bellies) occur in the Fynbos Biome but also extend into the Thicket Biome in the east of their distribution. The green-bellied *Z. virens* birds occupy mixed-leaved and broad-leaved Savanna, the eastern Grasslands, as well as fragmented forest patches found in the east of South Africa. The arid-adapted *Z. pallidus* (cinnamon-flanks and white-belly) is typically found in the fine-leaved Savanna, the Nama-Karoo and the dry, western Grassland, particularly along the wooded vegetation found along river courses in these later biomes. Typical *Z. senegalensis* (yellow-belly) birds are found in the drier Savanna in the northern parts of South Africa, extending into wetter Savanna and

montane Forest habitats. With the apparent restriction of these four southern African *Zosterops* taxa to particular Biome types, it is hypothesized that the observed phenotypic variation in the region may be driven by adaptations to particular habitats. It is however unlikely that biome type alone would be able to account for the variation observed. In this chapter, using a thorough sampling regime and by including five loci, it will be determined if there is any evidence of phylogeographic breaks between Biomes. As vegetation types are typically influenced by climatic conditions, the influence of environmental variables on phenotypic structure will also be explored.

Earlier morphological classification (Chapter 2), vocal analyses (Chapter 3) and a molecular phylogeny (Chapter 4) have produced interesting and unexpected inferences on southern African *Zosterops* evolutionary relationships. Of particular interest, *Z. senegalensis* has been shown to be sister to *Z. capensis* and *Z. virens* with *Z. pallidus* basal to these three taxa. However, hybridization and possible introgression has also been shown to occur between *Z. pallidus* and *Z. capensis*. Thus in addition, an attempt will be made to determine if further hybridization events have taken place, and particular with the use of mtDNA and nuDNA, to what extent introgression may have occurred.

5.3. Methods

5.3.1. Sampling

Blood and muscles tissue was collected from 371 individuals of the four southern African *Zosterops* colour forms: Orange River White-eye *Zosterops pallidus* (n = 97), Grey Cape White-eye *Z. capensis* (n = 148), Green Cape White-eye *Z. virens* (n = 116) and African Yellow White-eye *Z. senegalensis* (n = 10) from 45 localities throughout their distribution ranges (Fig. 5.1, Table 5.1). Samples were obtained from all seven South African Biomes: Forest (5 localities), Fynbos (6), Grassland (12), Nama-Karoo (5), Savanna (9), Succulent

Karoo (2) and Thicket (6). Sampling localities Aliwal North, Cookhouse, Graaf Reinet and Nova Vita fell on or near the junction between two biomes (Figure 5.1a, see Table 5.1 for details).

5.3.2. DNA extraction and PCR amplification

Genomic DNA was extracted from tissue and blood samples using either a standard phenol:chloroform extraction protocol or with the Qiagen DNeasy Kit (Qiagen, Valencia, California). Five molecular markers were used: mitochondrial ATP synthase 6 with a small flanking region of Cytochrome Oxidase subunit 3 (ATP6: 714 bp), autosomal introns Glyceraldehyde-3-phosphate Dehydrogenase intron-11 (GAPDH: 329 bp) and Transformation-growth-factor Beta 2 intron 5 (TGFβ2: 582 bp), as well as two sex-linked loci on the avian Z chromosome, Chromo-Helicase-DNA binding gene (CHD1: 476 bp) and Muscle Skeleton Receptor Tyrosine Kinase (MUSK: 571 bp). Double stranded DNA templates were PCR-amplified by the polymerase chain reaction (PCR). ATP6 was PCR-amplified using a standard protocol for 36 cycles at an annealing temperature of 54°C. The nuclear marker GAPDH was run for 37 cycles with an annealing temperature of 64°C, whereas TGFβ2 was amplified for 35 cycles at an annealing temperature of 58°C. The sex-linked loci CHD1 and MUSK were both amplified for 35 cycles at 50°C and 52°C, respectively. The same primers sequences as those used in the previous chapter (Chapter 4, Table 4.2) were used here. Three microlitres of PCR product was visualized on 1% Agarose gels, stained with ethidium bromide. The remaining PCR product was purified using 2.5ul of diluted ExoSAP solution incubated for 30 min at 37°C, and then for 15 min at 80°C. All purified products were sequenced using Big Dye terminator chemistry (Applied Biosystems, Foster City, CA, USA). Sequenced products were purified using Sephadex and then run on an AB3100 or AB3730 automated sequencer. All sequences were checked for insertions,

deletions or stop codons, and edited using the program BIOEDIT version 7.0.5.3 (Hall 1999).

5.3.3. Selection and recombination

DNASP ver. 5.10 (Librado and Rozas 2009) was used to test for selection acting on ATP6 using a MacDonald-Kreitman test (McDonald and Kreitman 1991) with sequences of *Z. erythropleura* and *Z. splendidus* as outgroups (choice of outgroups based on Moyle *et al*. 2009). To test for selection acting on the autosomal (GAPDH and TGFβ2) and sex-linked loci (CHD1 and MUSK) the HKA test (Hudson *et al.* 1987) as implemented in the program HKA (http://lifesci.rutgers.edu/~heylab/HeylabSoftware.htm) was used. The Single Breakpoint Recombination (SBP) and Genetic Algorithms for Recombination Detection (GARD) tests for intralocus recombination (Kosakovsky Pond *et al.* 2006) within each of the four nuclear loci was performed using DATAMONKEY 2010 (Kosakovsky Pond and Frost 2005, Delport *et al.* 2010).

5.3.4. Phasing of nuclear data

In order to infer genealogies and to estimate demographic parameters from molecular DNA sequence data it is necessary to resolve the phase of bi-allelic sequence data. All nuclear introns had more than one polymorphic site (SNP). A Bayesian method implemented in the program PHASE (Stephens *et al.* 2001, Stephens & Donnelly 2003) was used to resolve the phase of all linked polymorphisms. A conservative threshold of 0.75 (see Harrigan *et al*. 2008) was used to satisfactory consider all linked polymorphisms as phased; all individuals that did not meet this threshold were removed from further analyses, except for calculations of measures of genetic diversity.

5.3.5. Population genetic analyses

The number of polymorphic sites (P), the number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π) and Watterson's theta (θ) were calculated in DnaSP ver 5.10 for each putative species. TCS 1.21 (Clement *et al.* 2000) was used to reconstruct a 95% statistical parsimony network for each of the loci. Fu's *Fs* test (1000 replicates) and Ramos-Onsins and Rozas R^2 statistic (Ramos-Onsins and Rozas 2002), as implemented in DNASP ver. 5.10, was used to detect signatures of demographic change. Fu's *Fs* test has been shown to be a good indicator of population growth for large sample sizes ($n > 20$), whereas the R^2 statistic is a superior measure for smaller sample sizes (Ramos-Onsins and Rozas 2002). The significance of the R^2 statistic was assessed using 1000 coalescent simulations. An Analysis of Molecular Diversity (AMOVA) and Tamura and Nei pairwise F_{ST} (Tamura and Nei 1993) values among the four putative species (four morphogroups *sensu* Chapter 2) were calculated using ARLEQUIN ver. 3.11 (Excoffier *et al.* 2005). To test whether genetic structure could be better described/attributed by alternate population structure, additional AMOVAs were performed recognising three (*Z. pallidus*, *Z. capensis*/*Z. virens* and *Z. senegalensis*) and four phylogroups (*Z. pallidus*, *Z. capensis* (Western Cape localities only), *Z. capensis*/*Z. virens* and *Z. senegalensis*), respectively. These phylogroups are based on results from Chapter 4, which showed clear evidence of three distinct *Zosterops* taxa, and to a lesser extent evidence of *Z. capensis* individuals from the Western Cape being distinct from *Z. virens* and the other *Z. capensis* individuals sampled. To test whether biomes may have an important affect on shaping genetic structure, AMOVAs were also performed with Biome set as the grouping variable.

To test for the affect of geographic distance on genetic structure, isolation-by-distance was evaluated with a Mantel test with log transformed geographic distance (straight line distance between sampling localities) and Nei's genetic distance in GENALEX 6 (Peakall and

Smouse 2006). Mantel tests were done for *Z. pallidus*, *Z. capensis* and *Z. virens* separately for each of the five loci. As only two sampling localities were used for the collection of *Z. senegalensis* individuals, Mantel tests were not performed for this taxon. In addition, after partitioning the data by biome, Fu's *Fs* test and Ramos-Onsins and Rozas R^2 statistic were again used to detect signatures of demographic change, and isolation-by-distance analyses were performed to look at the affect of geographic distance on genetic structure. As only two sampling localities fell within the Succulent Karoo biome, isolation-by-distance analyses could not be performed between populations distributed in this biome.

A model-based Bayesian clustering method implemented in STRUCTURE, version 2.3.3 (Pritchard *et al.* 2000) was used to determine how individuals clustered when considering only the nuDNA data (autosomal and sex-linked). The length of the burn-in was set at 2×10^5 repetitions, followed by 2×10^6 MCMC repetitions. Individuals are assumed to have mixed ancestry (see Chapter 2), therefore the admixture model was used (Pritchard *et al.* 2000, Hubisz *et al.* 2009) and to assist in population clustering, a prior was placed on sampling locality (Hubisz *et al.* 2009). The remainder of the priors were left at the default values, *K* was set from 1 – 6, and 10 iterations per value of *K* were run. Output was modified using Distruct, version 1.1 (Rosenberg 2004).

5.3.6. Coalescent based estimates of gene flow and divergence times

The Bayesian Markov chain Monte Carlo method implemented in the program IMA (Hey & Nielson 2007) was used to fit the data to a model that included both isolation and migration. IMA estimates six parameters scaled to the neutral mutation rate (μ) and allows for likelihood-ratio tests to be performed between specific submodels of the six estimated parameters: θ_{Tax1} (4Neμ for Taxon 1), θ_{Tax2} (4Neμ for Taxon 2), θ_A (4Neμ for the ancestral

population at the time of divergence), *t* (T/μ, where T is the time since population divergence in years before present), m_{Tax1} (2M/θ_{Tax1}, where M is the effective number of migrants moving into Taxon 1 from Taxon 2) and m_{Tax2} (2M/θ_{Tax2}, the immigration rate into Taxon 2 from Taxon 1).

Inheritance scales were defined in the analyses using IMA to reflect the difference in modes of inheritance among the loci used: 0.25 for the mtDNA ATP6 gene (maternally inherited), 0.75 for the sex-linked loci (CHD1 & MUSK) and 1.0 for the two autosomal loci (GAPDH & TGFβ2; biparentally inherited) sequenced. A HKY model of mutation was used for all loci. IMA was first run to set appropriate upper priors for each parameter and to develop an appropriate heating scheme to help achieve efficient mixing of the eight Markov chains. The analysis was run for 2 x 10^7 generations, with a burn-in of 1 x 10^5 steps using geometric heating, with heating parameters of g1 = 0.15 and g2 = 0.7 and 24 chains. To assess convergence we monitored the extent of autocorrelation, parameter trend lines and the effective sample size (ESS) throughout the run, making sure that ESS values were at least above 50, as recommended in the IM and IMA documentation. An estimate of generation time and mutation rates is required to be able to convert the parameters estimated by IMA to biologically informative values. *Zosterops* are known to have a short generation time compared to that of other passerines (Moyle *et al.* 2009), therefore the default generation time of 1 year was used. A mutation rate of 2.475 x 10^{-8} substitutions/site/year (s/s/y) for mtDNA (4.95% per million years, Melo *et al* 2011) was used, which meant a per locus rate of 1.767 x 10^{-5} substitutions per year. A mutation rate of 3.6 x 10^{-9}s/s/y for the autosomal loci (Axelsson *et al.* 2004) was assumed. This translated into per locus rates of: CHD1 - 1.856 x 10^{-6}s/y, MUSK – 2.320 x 10^{-6}s/y, GAPDH – 1.184 x 10^{-6}s/y and TGFβ2 – 2.261 x 10^{-6}s/y. IMA analyses were performed on three pairwise data sets: *Z. virens* versus *Z. capensis*, *Z. virens* versus *Z. pallidus*, and *Z. virens* versus *Z. pallidus*. Due to time constraints (the average IMA

analysis would run for two months), IMA parameters could not be determined between *Z. capensis* and *Z. pallidus*. As a consequence the number of parameters were reduced and estimated using MDIV (Nielsen and Wakeley 2001). As *Z. senegalensis* is allopatric with respect to *Z. pallidus* and *Z. capensis*, pairwise comparisons were not performed between these taxa (Lee and Edwards 2008), but were performed between *Z. senegalensis* and *Z. virens*.

MDIV uses a Bayesian approach to estimate the posterior distribution of three parameters: θ (effective population size scaled to the mutation rate), M (migration rate between populations since divergence) and T (time of population divergence). The program was run for each of the loci used in the above IMA analyses, using the same prior settings where possible. The finite sites model (HKY model) was chosen, with the prior for migration rate set to 20 and the prior for divergence time set to 25. MDIV analyses were run for 1×10^7 generations following a burn-in period of 1×10^6 generations, and repeated twice with different random seeds to help ensure that convergence upon the same posterior distributions for each of the parameter estimates was achieved. Estimates of time of divergence (t_{div}) were converted to time before present using the formula: $t_{div} = (T * \theta)/2\mu$ (where μ is the per locus rate of substitutions per year used for the IMA analyses above). Credibility intervals (95% HPD) were calculated for θ and M, but not for T, as inaccurate credibility interval estimates are obtained due to the slow approach of T values to zero in the upper section of this curve (Johnson *et al.* 2007).

5.3.7. Environmental space analysis

To determine whether southern African *Zosterops* taxa occupy distinct climate envelopes as suggested by their apparent habitat affinities (Moreau 1957, Chapter 2), environmental data was collected for each of the sampling localities. High resolution (2.5 m)

bioclimatic data (www.worldclim.com) was imported into DIVA-GIS, version 7.1.7.2 and the 19 bioclimatic variables were extracted for each of the 43 sampling localities which occupy all seven Biomes within South Africa, and two sampling localities from montane forest in Malawi. A multi-dimensional scaling analysis was conducted on the bioclim data to determine potential multivariate grouping among localities. When grouping was observed among localities, a principle component analysis was performed to determine which environmental variables contributed to distinguishing among groups of localities.

5.4. Results

5.4.1. Selection and recombination

The McDonald and Kreitman test did not detect any evidence of selection within southern African *Zosterops* taxa (P = 0.357). Tajima's and Fu's *Fs* tests of neutrality were also conducted on the entire ATP6 dataset: both values were negative and not significant (Tajima's D: -0.317, P > 0.10; Fu's *Fs*: -1.658, P > 0.10). The HKA tests for neutrality of the autosomal and sex-linked loci were also not significant (all P > 0.10). No evidence of recombination was found for any of the nuclear loci using both SBP and GARD methods.

5.4.2. Mitochondrial DNA

The ATP6 region (714 bp) was amplified from 338 individuals, 130 from *Z. capensis*, 104 from *Z. virens*, 94 from *Z. pallidus* and 10 from *Z. senegalensis*. Mitochondrial DNA diversity measures show *Z. capensis* and *Z. virens* to have the highest haplotype and nucleotide diversity indices (Table 5.2). Pairwise genetic differentiation between *Z. pallidus* and the other taxa was high (*Z. capensis*: $F_{ST} = 0.719$, P < 0.001; *Z. senegalensis*: $F_{ST} = 0.793$, P < 0.001; *Z. virens*: $F_{ST} = 0.755$, P < 0.001). Genetic differentiation among the other taxa was

lower, but nonetheless significant (*Z. capensis* vs. *Z. senegalensis*: $F_{ST} = 0.282$, $P = 0.001$; *Z. capensis* vs. *Z. virens*: $F_{ST} = 0.129$, $P < 0.001$; *Z. senegalensis* vs. *Z. capensis*: $F_{ST} = 0.399$, $P < 0.001$). The large genetic differences between *Z. pallidus* and the other *Zosterops* taxa are evident in the ATP6 TCS parsimony network (Fig. 5.2), where *Z. pallidus* haplotypes are highly divergent from the other *Zosterops* haplotypes. Evidence of population expansion was detected in both *Z. capensis* (Fu's *Fs* = -15.74, $P = 0.001$; $R^2 = 0.056$, $P = 0.119$) and *Z. virens* (Fu's *Fs* = -33.75, $P < 0.001$; $R^2 = 0.044$, $P = 0.040$). No evidence of isolation-by-distance was observed among sampling points for any of the taxa ($R_{XY} = -0.075 – 0.185$, $P = 0.125 – 0.495$). No evidence of population expansion or isolation-by-distance was detected for any of the Biomes (Table 5.3).

The highest among-groups variation for mtDNA was obtained for three phylogroups ($\Phi_{CT} = 65.4$, $P < 0.001$), with very little difference in among-group variation between the four morphogroups and four phylogroups (Fig. 5.4). The Biome AMOVA shows that most variation is among-populations-within-groups ($\Phi_{SC} = 62.91$, $P < 0.001$; Fig. 5.4).

5.4.3. Nuclear DNA

5.4.3.1. CHD1

Complete sex-linked CHD1 (476 bp) sequences for 298 individuals (243 females and 55 males) were obtained for all four species. *Zosterops senegalensis* had the highest diversity indices, with other taxa showing low diversity values (Table 5.2). Pairwise F_{ST} values between *Z. senegalensis* and the other three taxa were similar (*Z. capensis*: $F_{ST} = 0.278$, $P < 0.001$; *Z. pallidus*: $F_{ST} = 0.276$, $P < 0.001$; *Z. virens*: $F_{ST} = 0.220$, $P < 0.001$), whereas differences among the other taxa were both low and non-significant (*Z. capensis* vs. *Z. pallidus*: $F_{ST} = 0.003$, $P = 0.175$; *Z. capensis* vs. *Z. virens*: $F_{ST} = 0.006$, $P = 0.057$; *Z. pallidus*

vs. *Z. virens*: $F_{ST} = 0.017$, P = 0.018). The TCS network revealed little structure within this locus (Fig. 5.3). All AMOVA analyses performed on CHD1 showed low among-group variation, with the majority of the variation being consistently attributed to within-populations (Fig. 5.4). There was evidence of population expansion for both *Z. capensis* (Fu's *Fs* = -10.99, P = 0.001) and *Z. virens* (Fu's *Fs* = -16.65, P < 0.001; $R^2 = 0.027$, P = 0.030). There was also evidence of population expansion within four biomes: Forest ($R^2 = 0.054$, P = 0.008), Grassland (Fu's *Fs* = -15.79, P < 0.001; $R^2 = 0.025$, P = 0.020), Nama-Karoo (Fu's *Fs* = -6.15, P = 0.006), and Savanna (Fu's *Fs* = -7.03, P = 0.004). No evidence of isolation-by-distance was observed for any of the putative taxa. Isolation-by-distance was only observed for the Thicket Biome ($R_{XY} = 0.629$, P = 0.046) (Table 5.3).

5.4.3.2. MUSK

Sequences from a total of 194 individuals were obtained for MUSK (bp = 596), the second sex-linked locus used in this study. Once again, *Z. senegalensis* had the highest genetic diversity for this locus (Table 5.2). There was also a relatively high degree of pairwise differentiation between *Z. pallidus* and the other taxa (*Z. capensis*: $F_{ST} = 0.630$, P < 0.001; *Z. senegalensis*: $F_{ST} = 0.696$, P < 0.001; *Z. virens*: $F_{ST} = 0.616$, P < 0.001). Differences between *Z. senegalensis* and both *Z. capensis* ($F_{ST} = 0.204$, P = 0.001) and *Z. virens* ($F_{ST} = 0.159$, P = 0.002) were significant. Significant genetic differentiation was observed between *Z. capensis* and *Z. virens* ($F_{ST} = 0.014$, P = 0.046). The Biome AMOVA results again indicate low among-group variation ($\Phi_{CT} = 1.88$, P = 0.290), whereas the three phylogroup AMOVA has the highest among-group variation ($\Phi_{CT} = 52.08$, P < 0.001; Fig. 5.4). The TCS network (Fig. 5.3) highlights this pattern to a certain extent, with *Z. pallidus* alleles grouping together, separated from the other species. Although *Z. senegalensis* has many unique alleles there is very little observed structure in the other taxa. No population expansion was observed in the MUSK

data for any of the *Zosterops* species, however, there is evidence of a population expansion in the Forest Biome (Fu's *Fs* = -4.982, P = 0.005; 0.069, P = 0.022). No evidence of isolation-by-distance was observed for any of the taxa or for any of the Biomes (Table 5.3).

5.4.3.3. GAPDH

A total of 354 individuals were sequenced for GAPDH (bp = 329). The genetic diversity was very similar among all species (Table 5.2), with Hd ranging from 0.669 to 0.762, π ranging from 0.0037 to 0.0040, although variation in θ was wider (0.0042 – 0.0072). Pairwise genetic differentiation between *Z. pallidus* and the other taxa is, although not particularly high, significant (*Z. capensis*: F_{ST} = 0.330, P < 0.001; *Z. senegalensis*: F_{ST} = 0.441, P < 0.001; *Z. virens*: F_{ST} = 0.460, P < 0.001). Although a much lower value, significant genetic differentiation is also observed between *Z. capensis* and *Z. virens* (F_{ST} = 0.033, P = 0.001). Low amounts of differentiation was also observed between *Z. senegalensis* and both *Z. capensis* (F_{ST} = 0.043, P = 0.039) and *Z. virens* (F_{ST} = 0.058, P = 0.017), whereas some semblance of structure is observed in the TCS network (Fig. 5.3). According to the AMOVA results most of the variation is within-populations across all analyses (Fig. 5.4), with the most among-group variation being explained by the three phylogroup analysis (Φ_{CT} = 36.13%, P < 0.001). Strong evidence of population expansion was observed in *Z. virens* (Fu's *Fs* = -8.27, P = 0.007), whereas weaker evidence was observed for *Z. capensis* (Fu's *Fs* = -4.29) and *Z. senegalensis* (Fu's *Fs* = -1.96, P = 0.054; R^2 = 0.09, P = 0.024). The Forest (Fu's *Fs* = -5.01, P = 0.006; R^2 = 0.05, P = 0.029), Savanna (Fu's *Fs* = - 5.36, P = 0.036) and Thicket (Fu's *Fs* = -5.01, P = 0.004) Biomes all showed evidence of population expansion. No evidence of isolation-by-distance was observed for any of the taxa or any of the Biomes (Table 5.3).

5.4.3.4. TGFβ2

Complete sequences of TGFβ2 for 371 individuals were obtained, with genetic diversity greatest in *Z. senegalensis* (Table 5.2). Again, relatively high and significant pairwise genetic differentiation was observed between *Z. pallidus* and the other taxa (*Z. capensis*: F_{ST} = 0.535, P < 0.001; *Z. senegalensis*: F_{ST} = 0.625, P < 0.001; *Z. virens*: F_{ST} = 0.576, P < 0.001). Slightly lower, but still significant, levels of genetic differentiation was observed between *Z. senegalensis* and *Z. capensis* (F_{ST} =0.563, P < 0.001), and *Z. virens* (F_{ST} = 0.457, P < 0.001); low but significant levels of genetic differentiation was detected between *Z. capensis* and *Z. virens* (F_{ST} = 0.057, P < 0.001). The three phylogroup AMOVA once again accounted for the highest among-group variation (Φ_{CT} = 50.06%, P < 0.001; Fig. 5.4), whereas little difference in among-group variation was shown between the four morphogroup and four phylogroup analyses (Fig. 5.4). The Biome AMOVA again accounted for low among-group variation (Φ_{CT} = 13.14, P = 0.040). There was no evidence of population expansion for any of the *Zosterops* species, but there was for three Biomes: Forest (Fu's *Fs* = -5.15, P = 0.048), Succulent Karoo (Fu's *Fs* = -3.36, P = 0.047, R^2 = 0.04, P = 0.050), and Thicket (Fu's *Fs* = -3.94, P = 0.001). No evidence of isolation-by-distance was observed for any of the taxa or any of the Biomes (Table 5.3).

5.4.4. Population structure

Consistent STRUCTURE results were obtained for value of *K* from 1 – 6. The probability of the data (LnPr L(*K*)) was maximum at *K* = 5, however similar probability levels were attained for *K* = 6 (Fig. 5.5a), although the standard deviation at this *K* value was high (SD = 86.4). Following the methodology of Evanno *et al*. (2005), the highest deltaK (ΔK) value is attained at *K* = 2 (Fig. 5.5b). At this value of *K*, *Z. pallidus* forms a distinct group with the other white-eye taxa grouping together, although some individuals of *Z.*

capensis and *Z. virens* do share nuDNA with *Z. pallidus* (Fig. 5.6). The addition of further clusters (K = 3, 4 & 5) increases the probability of the data (Fig. 5.5a), with *Z. senegalensis* individuals forming a separate cluster to *Z. capensis* and *Z. virens*. Only at K = 5 do *Z. capensis* and *Z. virens* show some level of separation from each other (Fig. 5.6).

5.4.5. Gene flow and population divergence

5.4.5.1. Z. capensis *and* Z. virens

The estimated geometric mean of mutation rate among the five loci (mtDNA and nuDNA) was 2.44 x 10^{-6} s/l/y. Using an average generation time of one year, IMA suggests a N_e (Fig. 5.9a) of 1 512 889 individuals for *Z. virens* (90% HPD = ~ 1 060 000 – 2 480 000), and 505 994 individuals for *Z. capensis* (90% HPD = ~ 359 000 – 689 000). The N_e of the ancestral population of *Z. capensis* and *Z. virens* is inferred to be 531 415 (90% HPD = ~ 170 358 – 4 116 578) individuals (Fig. 5.7a). The large confidence interval of the 90% HPD of the ancestral population indicates that these values may not be reliable. Interestingly, a nested model showing similar population size of *Z. capensis* and the ancestor could not be rejected (log (P) -6.3694, df = 1, ns) using a likelihood-ratio test. All nested models with *Z. virens* and ancestral populations having similar populations sizes could be rejected ($P < 0.05$). This result, together with strong evidence for expansion of *Z. virens* at most loci, suggests that *Z. capensis* has maintained a relatively small stable population size, whereas *Z. virens* has expanded since these taxa diverged.

Contrasting values of M were estimated for *Z. capensis* and *Z. virens* (Fig. 5.7d), and as such migration rates between the two taxa differed considerably. *Zosterops virens* alleles enter *Z. capensis* at a mean rate of 3.47 (90% HPD = 0.41 – 18.78), whereas the mean rate at which *Z. capensis* alleles enter *Z. virens* is considerably higher at 63.16 (90% HPD = 0.03 –

120; the multiple peaks for this posterior density curve make this HPD estimate unreliable though). A model of asymmetrical gene flow could not be rejected (log(P) – 6.3694, df = 1, ns). All nested models with zero migration rates between *Z. capensis* and *Z. virens* were rejected using the likelihood-ratio test, however, the model with different N_e between θ_{cap}, θ_{vir} and $\theta_{ancestral}$ but with migration into *Z. capensis* and no migration into *Z. virens* was not rejected (log (P) -6.0682, df = 1, ns). The MDIV migration estimates also showed high levels of gene flow between *Z. capensis* and *Z. virens* (Table 5.4) for all loci.

The IMA posterior density curve for *t* was unimodal (Fig. 5.7g), with a mean value of 0.1375, translating to a divergence time of 56 000 ya BP (90% HPD = 25 600 – 4 830 000), however the confidence interval is considerable, and should be used with caution especially as the upper value pre-dates the diversification of *Zosterops* in Asia ~ 1.40 – 1.89 mya (Warren *et al.* 2006, Moyle *et al.* 2009). MDIV estimates of divergence dates between *Z. capensis* and *Z. virens* fall within the 90% HPD determined by IMA, ranging from 57 353 – 174 560 ya (Table 5.4), although the divergence estimate for GAPDH varied considerably across all replicate runs. Performing longer coalescent runs for this locus may overcome the possibility of insufficient information and produce more robust time estimates.

5.4.5.2. Z. virens *and* Z. pallidus

When comparing *Z. virens* and *Z. pallidus* populations using IMA, and using the same geometric mean of mutation rate as above, the N_e of *Z. virens* and *Z. pallidus* is estimated to be 538 535 (90 HPD: ~ 177 510 – 1 296 700) and 39 110 (90% HPD: ~ 21 055 – 117 336) individuals, respectively (Fig. 5.7b). The ancestral N_e between these taxa is estimated at 111 322 (90% HPD: ~ 3 012 – 4 828 800) individuals, although once again the large 90% HPD of the ancestral population suggests that caution should be used as these values may be unreliable (Fig. 5.7b). Likelihood-ratio tests reject most models where daughter population

sizes are equal, or where either daughter population is equal to the ancestral population, except for two models where *Z. pallidus* population size equals the ancestral population size (log (P) -4.0606, df = 1, ns; log (P) -4.0419, df = 2, ns).

Values of *M* for *Z. pallidus* and *Z. virens* were very similar (Fig. 5.7e), however, with contrasting N_e for each population, this translated into differing allele migration rates. The mean migration rate of alleles into the *Z. virens* from *Z. pallidus* (5.30; 90% HPD: 3.19 – 56.41) is much higher than the mean rate into *Z. pallidus* from *Z. virens* (0.54; 90% HPD: 0.29 – 1.61). All nested models with zero migration could be rejected, as well as most models with asymmetrical migration. Models with equal migration rate between taxa, and either different effective population sizes (log (P) = -1.2542, df = 1, n.s) or equal effective population sizes of *Z. pallidus* and the ancestral population (log (P) = -4.0419, df = 2, ns) could not be rejected. Evidence of gene flow between *Z. virens* and *Z. pallidus* is also found in the MDIV analysis (Table 5.4), and whereas most loci showed low M values, CHD1 was relatively high (*M* = 3.22).

The time of divergence between *Z. virens* and *Z. pallidus* (Fig. 5.7g), estimated by IMA, occurred 35 860 ya (90% HPD: 5 123 – 5 527 663). The 90% HPD interval is again large, making this divergence estimate unreliable. The MDIV divergence estimates (Table 5.4) also do not provide a clear indication of divergence time, with estimates from GAPDH and TGFβ2 (> 3.3 mya) pre-dating global *Zosterops* diversification (Warren *et al.* 2006, Moyle *et al.* 2009).

5.4.5.3. Z. capensis *and* Z. pallidus

Time constraints prevented the running of the IMA analysis of *Z. capensis* and *Z. pallidus*. Migration and divergence date parameters between these taxa were however

obtained from the MDIV analyses. Estimates of migration suggest that gene flow occurs between Z. *capensis* and *Z. pallidus*, albeit at low levels for most loci (Table 5.4), however, values of migration for CHD1 was once again high ($M = 6.56$). Similar divergence date estimate issues and patterns were encountered for *Z. capensis* and *Z. pallidus* (Table 5.4) as well as for those estimates of *virens*/*pallidus* comparisons. Only the two sex-lined loci, MUSK and CHD1, show divergence estimates that fall after global *Zosterops* diversification (Table 5.4), whereas the estimate of GAPDH falls at the lower end of the confidence interval of that diversification event (Moyle *et al.* 2009).

5.4.5.4. Z. virens *and* Z. senegalensis

Population size estimates of *Z. virens* and *Z. senegalensis*, as estimated by IMA, are 1 557 275 (90% HPD: 984 345 – 2 581 230) and 1 100 145 (90% HPD: 868 535 – 1 392 705) individuals, respectively (Fig. 5.7c). The ancestral population size is estimated to be 575 975 (90% HPD: 277 325 – 1 069 670) individuals, much lower than the two daughter populations. All likelihood ratio tests between *Z. virens* and *Z. senegalensis* could not be rejected ($P > 0.05$), thus population sizes of *Z. virens*, *Z. senegalensis* and the ancestral population may be similar.

Migration rates between both taxa were low (Fig. 5.7f). The mean migration rate of alleles moving from *Z. senegalensis* into *Z. virens* was 0.49 (90% HPD: 0.02 – 4.85), with that of *Z. virens* into *Z. senegalensis* at 0.03 (90% HPD: 0.02 – 0.92). Again, as none of the nested models could be rejected, scenarios of zero and asymmetrical migration rates could be equally likely. Additionally, MDIV migration rates estimates were also low across all loci (Table 5.4).

The divergence time estimate from IMA places the divergence of *Z. virens* and *Z.*

senegalensis at around 790 000 ya (90% HPD: 353 485 – 1 193 650, Fig. 5.7g). The divergence time estimate for TGFβ2 is very high, while the other loci all estimate the split between *Z. virens* and *Z. senegalensis* at less than 1 mya (Table 5.4).

5.4.6. Environmental space

The environmental variables tend to group collecting localities by putative taxon. Localities that fall on the boundary between the ranges of *capensis* and *virens* (Grahamstown and Kasouga) fall in the zone between these taxon localities (Fig. 5.8a). Sampling sites where both *Z. pallidus* and *Z. capensis* were collected (Aliwal North, Rouxville, Taba Nchu, and especially Nova Vita) tended to group closer to typical *Z. pallidus* localities (Fig. 5.8b). This trend is not followed between *Z. pallidus* and *Z. virens* mixed localities, with Vanderbijlpark and Lindley falling between pure sampling localities of each taxon. *Zosterops senegalensis* sampling sites (Nyika and Zomba) grouped closely with northeasterly *Z. virens* localities (Louis Trichard and Magoebaskloof).

The first principle component accounted for 78.6% of the variation among localities, separating eastern sampling localities from those in the west. Two variables accounted for the majority of the variation: temperature seasonality (0.741) and annual precipitation (-0.530). The second principle component (13.6 % variation) separated *Z. capensis* localities (barring two *Z. virens* localities: Kasouga and Morgan Bay) from the localities where other taxa occur. Precipitation of the coldest quarter (-0.635) and precipitation of the wettest quarter (0.435) were the main contributors to this variation.

5.5. Discussion

5.5.1. Genetic structure and Biome affects

A molecular phylogeny of southern African *Zosterops* taxa (Chapter 4) incorporating samples from the core of the distribution range of each of the taxa revealed surprising relationships among the four main plumage colour forms (Moreau 1957, Clancey 1967, Skead 1967, Chapter 2). With more comprehensive sampling from all of South Africa's biomes, and especially with the inclusion of sampling localities at the edge of the range for each of the four morphologically distinct *Zosterops* taxa, a greater understanding of the processes underpinning the spatial structure of these taxa and associated demographic histories has been obtained.

The patterns of genetic structure, revealed by statistical parsimony networks and F_{ST} values are collectively similar to those patterns produced with a smaller sample size (Chapter 4). To briefly revisit this genetic structure, mtDNA revealed distinct and divergent *Z. pallidus* haplotypes (Fig. 5.2a); whereas a lack of predictable geographic groupings were observed in the *capensis*/*senegalensis*/*virens* mtDNA network (Fig. 5.2b). Similarities in plumage colouration between *Z. senegalensis* and *Z. virens* together with their parapatric distribution should see these taxa grouping closer together. While this network shows congruence with the molecular phylogeny from Chapter 4 (*Z. senegalensis* sister to *Z. capensis* and *Z. virens*), it would be expected that *Z. senegalensis* haplotypes would group more closely with *Z. virens* than with haplotypes sampled from western localities of *Z. capensis'* distribution.

With the exception of CHD1, the nuclear loci showed fairly concordant patterns of *Zosterops* population structure with that of the mtDNA data (Fig. 5.3). As a whole, the nuclear loci showed allele sharing and were less divergent among taxa relative to the mitochondrial data which showed *Z. pallidus* to be sister to the other *Zosterops* taxa. This discordance is expected and is, in part, due to the higher effective population size of nuDNA

in comparison to that of mtDNA, as well as to a higher mtDNA mutation rate compared to that of nuDNA (Moore 1995, Hare 2001, Zink and Barrowclough 2008).

The distribution of CHD1 alleles among the four taxa is in striking contrast to that observed for the other nuclear loci as well as for the mitochondrial data, with *Z. senegalensis* alleles being divergent from the other taxa (Fig. 5.3; $F_{ST} > 0.22$, $P < 0.001$). Positioning of the CHD1 gene on the Z-chromosome may be a factor in the contrasting genetic structuring observed. The position of CHD1 is fairly close to the centromere of the sex-chromosome of the chicken (Itoh *et al.* 2006), the Zebra finch (Itoh *et al.* 2006) and the Collard flycatcher (Backström *et al.* 2008) and given the similarity of the CHD1 position across all taxa, here a similar position in *Zosterops* is assumed. In contrast, the position of the other sex-linked locus, MUSK, on the Chicken genome is toward the telomeric end of the Z-chromosome (http://www.ncbi.nlm.nih.gov/genome/guide/chicken/). The position of the MUSK gene for other species has not been determined as yet; hence a similar position for *Zosterops* taxa is assumed. Genes closer to the centromere have been shown to have lower levels of genetic variability compared to genes closer to the telomeres (Geraldes *et al.* 2006), hence the possible lack of structure observed here among taxa. This however, does not explain the observed divergence of *Z. senegalensis* alleles. As discussed in more detail below, the mtDNA and nuDNA data sets reveal hybridization of *Z. pallidus* with both *Z. capensis* and *Z. virens*. Thus, high levels of CHD1 introgression is possibly negating the faster adaptive rate of divergence of Z-linked loci (Charlesworth *et al.* 1987; Mank *et al.* 2007, 2009; Ellegren 2009, Elgvin *et al.* 2011), potentially explaining the lack of structure between these three taxa.

Each of the four phenotypically distinct *Zosterops* taxa is generally found to occur in distinct Biomes in southern Africa, and as such exploring the role of Biomes in shaping diversity can highlight evolutionary processes in the region. Very little among-group

variation for any of the molecular data was explained using Biomes as a grouping. Grouping the molecular data by three phylogroups (*Z. pallidus*, *Z. capensis*/*virens* and *Z. senegalensis*; *sensu* Chapter 4) however, consistently accounted for the most among-group variation. Thus, historical divergence (Chapter 4) is likely to have shaped *Zosterops* diversity with the Biome preferences of each taxon arising relatively recently and may yet substantially affect the genetic structure of these birds. Given the poor Biome F_{ST} values, environmental variables do appear to impact the current distribution of white-eyes in southern Africa with precipitation measures being important explanatory variables. While the neutral markers in this study have yet to be impacted and have failed to reveal any distinct phylogeographic breaks corresponding to environmental and habitat conditions, local adaptation and phenotypic variation may already be influenced by these varying environmental conditions.

Population expansion was consistently observed for the Forest biome in the nuDNA data, however this is potentially misleading as Forests in South Africa are highly fragmented and scattered (Midgley *et al*. 2004), being found in the southwest (*Z. capensis*) and northeast (*Z. virens*) of the region, as well as in two localities in Malawi (*Z. senegalensis*). Forests in South Africa have expanded since the Last Glacial Maximum (~ 18 000 ya; Eeley *et al*. 1999, Lawes *et al*. 2007), thus possibly explaining the population expansion revealed here. There was no consistent evidence for expansion of *Zosterops* from the other Biomes across the molecular data.

5.5.2. Combined nuclear data, gene flow and hybridization

Two mechanisms appear to be at play regarding allele and haplotype sharing between the southern African white-eyes. The first, incomplete lineage sorting is likely to explain the sharing of nuDNA between *Z. senegalensis* and *Z. virens* (and to a lesser extent *Z. capensis*). The increase in the values of *K* in the STRUCTURE analysis demonstrate that *Z. senegalensis*

individuals formed a definitive group (as opposed to when $K = 2$), however nuDNA sharing with *Z. virens* is still observed. Together with a fairly recent time since divergence (~ 300 000 ya; Chapter 4), coalescent analyses between *Z. senegalensis* and *Z. virens* reveal that gene flow is not significantly different from zero. Thus, insufficient time has passed for these two white-eye taxa to have become reciprocally monophyletic, particularly with regard to nuDNA.

Secondly, secondary contact and introgressive hybridization is likely to be responsible for allele and haplotype sharing between *Z. pallidus*, *Z. capensis* and *Z. virens*. Although coalescent analyses reveal recent divergence times between *Z. pallidus* and *Z. virens*, the reliability of the timing of migration results from IM and IMa has been questioned from simulation studies (Becquet and Przeworski 2009, Strasburg and Rieseberg 2011). Here, recent divergence times are in conflict with other divergence time estimates for southern African white-eyes (~ 770 000 ya; Chapter 4), with the results presented here likely to have been influenced by recent introgression (Peters *et al.* 2007, Strasburg and Rieseberg 2011).

The difficulty of obtaining convergence of the parameter estimates produced by IMA has been widely reported (Peters *et al.* 2005, Garrigan *et al.* 2007, Bonvicino *et al.*2009, Bowie *et al.* 2009). Although not infallible, monitoring ESS values during IMA analyses can be used to assess parameter convergence, with values greater than 50 recommended as the minimum (IM and IMA manual). Obtaining the same parameter estimates across multiple runs using the same priors is also indicative of parameter convergence. In this study, all ESS values were above 50, although the *t* parameter was below 100. All other values were greater than 200. This is indicative of the large variance around the *t* estimate reported above, and therefore all divergence time estimates reported from the IMA analyses are interpreted with caution.

Unfortunately, time constraints prevented multiple runs being performed, however,

multiple runs will be performed in future work in order to obtain robust parameter estimates. An increase in the number of loci used in the IMA analyses may also allow for better convergence (Lee and Edwards 2008). Incorporating MDIV pairwise species comparisons with the IMA results has increased confidence in establishing gene flow between taxa, however divergence time estimates have been unreliable for some of the loci used. Species tree divergence time estimates (Chapter 4) removed any biases that hybridization and introgression may produce and provided more robust divergence dates. All taxa shared a common ancestor ~ 770 000 ya, after which *Z. pallidus* split from the other taxa. The split between *Z. senegalensis* and both of *Z. capensis* and *Z. virens* occurred ~ 300 000, whereas low support values do not provide confidence in estimating the split between *Z. capensis* and *Z. virens*.

No evidence of hybridization is observed in any of the individuals from the *Z. pallidus* population. Thus, introgression appears to be occurring from *Z. pallidus* into individuals of *Z. capensis* and *Z. virens*. All coalescence analyses indicate that there is migration between these taxa, and although the direction of migration cannot be determined using the MDIV analyses, the IMA analysis of *Z. pallidus* and *Z. virens* shows high rates of migration into the *Z. virens* population, but low rates in the opposite direction. With the inability to complete the IMA analysis between *Z. capensis* and *Z. pallidus*, a firm estimate of migration rates between the two populations cannot be determined. However, a similarity of migration rates between *Z. pallidus* and *Z virens*, and a lack of *Z. capensis* nuclear DNA found in *Z. pallidus* individuals, it is presumed that substantial gene flow from *Z. pallidus* is occurring into the *Z. capensis* population.

Hybridization and introgression was observed in the mtDNA as well as in the combined nuDNA data sets. Phenotypically pure *Z. capensis* (n = 9) and *Z. virens* (n = 7) individuals from Aliwal North, Lindley and Vanderbijlpark (all localities on contact with *Z.*

pallidus) were introgressed at the mtDNA, possessing *Z. pallidus* haplotypes. Introgression of mtDNA does however, not appear to occur in just the one direction. Four phenotypically pure *Z. pallidus* individuals grouped with mainly *Z. capensis* and *Z. virens* haplotypes. Three individuals were from Aliwal North (contact locality with *Z. capensis*), whereas the other was from Vioolsdrift, a purely *Z. pallidus* locality.

Introgression of nuclear loci was observed at all levels of population structure (i.e. $K = 2$ up to $K = 5$), with the same individuals consistently possessing high *Z. pallidus* membership (> 40%). These phenotypically pure individuals (either *Z. pallidus* or *Z. virens*) were collected from localities of overlap with *Z. pallidus* (Aliwal North, Nova Vita, Lindley, and Vanderbijlpark). Two *Z. capensis* individuals from outside of the *Z. pallidus* contact zones (Kamiesberg and Tarkastad) also showed evidence of genetic introgression, indicating that the extent of introgression may extend further that just within localities of contact (Rohwer *et al*. 2001).

5.5.3. Taxon histories and inferences about southern African biogeography

The predominant focus of southern African phylogeographic research has centred on taxa distributed in the southwestern areas of the region. Congruent patterns of biogeographic breaks have been described in many studies, particularly the effects of the Knersvlakte on dividing southern and northern groups (i.e. Matthee and Robinson 1996, Matthee and Flemming 2002, Smit *et al*. 2007, Portik *et al.* 2011), or the Cape Fold Mountains dividing western and eastern groups (i.e. Daniels *et al*. 2007). Yet, the structure of other taxa appears to have been influenced by mountain refugia or habitat/biome type (i.e. Tolley *et al*. 2006, 2008, 2010; Swart *et al*. 2009; Smit *et al*. 2010). Most of these evolutionary theories have been applied to taxa with low vagility, and are thus more likely to be under the influence of vicariant processes. Thus, it is interesting that in a highly mobile and generalist passerine,

southern African *Zosterops* diversity appears to be shaped by vicariant/historical divergence. This study has revealed no strong affect of habitat or climate on genetic diversity, although recent phenotypic adaptation to climate, not yet revealed in neutral genetic data, appears to have taken place.

5.5.3.1. Zosterops pallidus

The Orange River White-eye is distributed in the arid central and western areas of southern Africa, though individuals are restricted to river courses and human habitation. With no evidence of range expansion or isolation-by-distance for any of the molecular loci, the historical population distribution range is expected to be similar to that seen at present. Three putative subspecies (*Z. p. sundevalli*, *Z. p. deserticola* and *Z. p. haigamchabensis*) of the Orange River White-eye have been proposed (Bowie 2005), however, the lack of genetic structure presented here strongly suggests that *Z. pallidus* is monotypic. River courses are expected to have facilitated movement of individuals throughout the range of *Z. pallidus*, accounting for the lack of structure observed within this taxon.

Morphometric, vocal and the taxon's placement in a molecular phylogeny (Chapter 4) strongly suggest the recognition of *Z. pallidus* as a valid species, distinct from the other southern African *Zosterops* taxa. Genetic diversity statistics do not deviate from this view. Hybridization between *Z. pallidus* and *Z. virens* has been reported in the past (Clancey 1967, Skead 1967), with individuals of intermediate plumage colouration being found in zones of contact (Chapter 2). Genetic evidence presented here (Fig. 5.2 & 5.6) together with the coalescent gene flow estimates (Fig. 5.7) indicate gene flow between *Z. pallidus* and both *Z. capensis* and *Z. virens*, with migration rates indicating introgressive hybridization into *Z. virens*. Results from the STRUCTURE analysis (Fig. 5.6) also suggest introgressive hybridization is occurring from *Z. pallidus* into *Z. capensis*. Secondary contact and

subsequent hybridization is likely to have affected divergence time estimates reported from the IMA coalescent analysis between *Z. pallidus* and *Z. virens*. Divergence time estimates have been shown to be more recent between introgressed populations than between sympatric populations that have been in isolation but with migration between populations (Peters *et al.* 2007). Removing hybrid individuals identified in the STRUCTURE analyses will provide better estimates of effective population sizes as well as determine whether species tree divergence estimates generated in Chapter 4 (~ 770 000 ya) are similar to IMA coalescent estimates for these two populations.

With hybridization events across contact zones with both *Z. capensis* and *Z. virens*, as well as indications of range expansion for both of these taxa and no genetic structure within *Z. pallidus*, it is likely that the historical distribution range of the Orange River White-eye is very similar to what we observe at present.

5.5.3.2. Zosterops capensis *and* Zosterops virens

Plumage differences between *Z. capensis* and *Z. virens* have been addressed previously (Chapter 2), with underpart plumage colouration proving to be a diagnostic character between these two taxa. While gene flow between populations is expected to have a homogenizing effect on colour polymorphisms, some systems show that this is not always the case due either to divergence in allopatry with limited gene flow upon secondary contact (Cooke *et al.* 1988, Lim *et al.* 2010), due to social and ecological constraints preventing complete isolation (Pryke 2010), or due to local adaptation (Antoniazza *et al.* 2010). A lack of corroboration between *Zosterops* plumage polymorphism and the neutral genetic data, as well as high levels of gene flow observed, suggest that a single or a few genes, local adaptation, strong divergent selection or a recent origin of plumage differences (Mila *et al.*

2010) could be responsible for *Zosterops* plumage colouration observed here. Other *Zosterops* taxa have shown the propensity to undergo rapid changes in body size when colonizing islands (Clegg *et al*. 2002, Clegg *et al.* 2008), while gene flow between *Zosterops* populations has not prevented phenotypic divergence between taxa (Clegg and Phillimore 2008, Milá *et al.* 2010).

The adaptation of dispersing individuals to the novel Fynbos Biome may have driven the morphological differences observed between *Z. capensis* and *Z. virens*. As there is no obvious geographic barrier separating these two taxa, adaptation to contrasting environmental and climatic conditions could have caused changes in plumage colouration and subsequently begun the process of diversification or ecological speciation. Defined as achieving reproductive isolation between populations or subsets of populations due to ecologically-based natural selection (Schluter 2001, Rundle and Nosil 2005, Schluter and Conte 2009), ecological speciation has been shown to occur in sympatry (Feder *et al.* 2003), however is more likely, in this case, to have occurred in parapatry. Divergence between *Z. capensis* and *Z. virens* is likely to have been facilitated by contractions of forest during dry cycles of the Pleistocene (Eeley *et al*. 1999, Lawes *et al*. 2007). Categorically establishing those ecological factors that have been important in the divergence of these two *Zosterops* taxa is difficult, but further work on gene expression (Pavey *et al.* 2010, Wolf *et al.* 2010) is likely to shed light on the factors important for ecological speciation in these taxa.

Zosterops are described as having generalist behaviour and feeding habits (Gill 1936, Roberts 1942, McLachlan & Liversidge 1978, Fry *et al.* 2000), thus local feeding and/or behavioural adaptations to novel habitats (i.e. Fynbos biome) would be seen to account for a distinct evolutionary lineage beginning to emerge in the mtDNA data. A more likely influence on *Zosterops* speciation would have been changes in the timing of rainfall in the western areas of southern Africa, to that of a winter rainfall zone (Franz-Odendaal *et al.* 2002). Moult

and breeding among different *Zosterops* populations in southern Africa are correlated (Hulley *et al.* 2004), with individuals within the present winter rainfall zone beginning moult (Earlé 1981; Craig 1983, 1990; Dowsett 1985, Whitelaw 1985, Symes *et al.* 2001) and breeding (Nuttal 1997) earlier than those individuals in the year-round and summer rainfall zones. The fixation of an earlier breeding season for individuals in the winter rainfall zone could facilitate reproductive isolation between winter and summer rainfall zone individuals.

The presence of gene flow between *Z. capensis* and *Z. virens* is not unexpected as mixed pairs and evidence of hybrid individuals in contact areas have been recorded (Skead 1967), while similarities in song (Chapter 3) would not preclude these taxa from interbreeding. Cycles of warm, wet and cool, dry periods every 100 000 years over the past 600 000 years (Marlow *et al.* 2000) would have shifted the ranges of the winter and summer rainfall zones over this period and allowed the maintenance of genetic cohesion between the two colour forms. These climatic cycles could have also facilitated the population expansion of each taxon observed in some of the molecular markers (ATP6, CHD1 and GAPDH), with these expansion events possibly resulting in the secondary contact and subsequent hybridization of both these populations with *Z. pallidus* in contact zones in the southern and eastern Free State. Future research aims will be to establish behavioural and environmental factors facilitating hybridization between these taxa, through analyses of vocal behaviour and physiological adaptation within zones of contact.

5.5.3.3. Zosterops senegalensis

Although the sample size of *Z. senegalensis* individuals used in this study is relatively small (n = 10), and restricted to two sampling localities in Malawi (Table 5.1; Fig. 5.1), their inclusion has allowed the confirmation of the taxonomic relationship of southern African

Zosterops taxa. Ecological differences have been hypothesised to separate *Z. senegalensis* and *Z. virens* (Moreau 1957), with no records of interbreeding between them. The coalescent-based analyses support this view, revealing low levels of gene flow between *Z. senegalensis* and *Z. virens* (Table 5.2). These levels may however, be an underestimate of true migration as populations of *Z. senegalensis* parapatric to *Z. virens* are at present unsampled.

Table 5.1: Sampling localities, GPS coordinates and number of samples for each locus used in the phylogeographic analysis of the four southern Africa *Zosterops* taxa: *Zosterops capensis*, *Z. pallidus*, *Z. senegalensis* and *Z. virens*.

Species	Locality	Biome	GPS	ATP6	CHD1	MUSK	GAPDH	TGFβ2
Z. capensis	Aguhlas NP (ANP)	Fynbos	34° 45' 46.78" S, 19° 55' 38.64" E	2	2	2	2	2
Z. capensis	Aliwal North (AN)	Nama-Karoo/Grassland	30° 39' 24.42" S, 26° 32' 49.08" E	10	12	10	10	12
Z. capensis	Anysberg NP (ANY)	Succulent Karoo	33° 27' 42.27" S, 20° 35' 04.05" E	2	2	2	1	2
Z. capensis	Beaufort West (BW)	Nama-Karoo	32° 19' 54.00" S, 22° 45' 46.00" E	6	7	3	7	7
Z. capensis	Bontebok NP (BNP)	Fynbos	34° 04' 40.00" S, 20° 27' 14.00" E	7	8	2	6	7
Z. capensis	Cape St Francis (CSF)	Thicket	34° 11' 03.56" S, 24° 48' 53.38" E	1	1	1	-	1
Z. capensis	Cape Town (CT)	Fynbos	33° 59' 21.98" S, 18° 41' 46.26" E	18	12	1	16	21
Z. capensis	Clarens (CLR)	Grassland	28° 31' 05.78" S, 28° 25' 18.95" E	3	2	2	2	2
Z. capensis	Cookhouse (CH)	Nama-Karoo/Grassland/Savanna	32° 44' 59.93" S, 25° 48' 59.72" E	2	2	-	2	3
Z. capensis	De Rust (DR)	Fynbos	33° 35' 50.00" S, 22° 31' 37.00" E	14	3	2	15	16
Z. capensis	Dordrecht (DOR)	Grassland	31° 22' 29.45" S, 27° 02' 51.52" E	4	2	1	5	3
Z. capensis	Graaf Reinet (GR)	Thicket/Grassland/Nama-Karoo	32° 15' 00.40" S, 24° 32' 07.44" E	2	2	-	3	3
Z. capensis	Grahamstown (GHT)	Thicket	33° 21' 53.78" S, 26° 31' 43.41" E	1	1	1	1	1
Z. capensis	Grootvadersbosch (GVB)	Forest	33° 59' 03.01" S, 20° 49' 33.43" E	2	1	-	-	-
Z. capensis	Kamiesberg (KMB)	Fynbos	30° 22' 24.40" S, 18° 05' 57.00" E	7	7	3	10	8
Z. capensis	Nova Vita (NV)	Nama-Karoo/Fynbos	32° 33' 27.06" S, 21° 25' 58.62" E	33	31	33	30	33
Z. capensis	Picketberg (PKB)	Fynbos	32° 55' 48.35" S, 18° 45' 58.39" E	2	1	2	3	3
Z. capensis	Port Elizabeth (PE)	Thicket	33° 41' 57.65" S, 25° 18' 36.81" E	-	-	-	2	-
Z. capensis	Rouxville (RV)	Grassland	30° 24' 46.94" S, 26° 49' 57.00" E	1	-	-	1	1
Z. capensis	Storms River (SR)	Forest	33° 58' 50.07" S, 23° 56' 38.71" E	5	1	-	-	-
Z. capensis	Taba Nchu (TN)	Grassland	29° 16' 15.13" S, 26° 54' 22.41" E	3	3	3	3	3
Z. capensis	Tarkastad (TKS)	Grassland	32° 00' 52.53" S, 26° 16' 07.88" E	18	21	20	19	20
Z. pallidus	Aandster (AS)	Savanna	27° 28' 51.44" S, 25° 11' 12.75"E	-	1	1	1	1
Z. pallidus	Aliwal North (AN)	Nama-Karoo	30° 39' 24.42" S, 26° 32' 49.08" E	9	9	9	8	9
Z. pallidus	Bloemfontein (BFT)	Grassland	29° 08' 28.60" S, 26° 13' 17.53" E	2	2	-	2	2
Z. pallidus	Brabeesmond (BBM)	Nama-Karoo	28° 38' 59.99" S, 20° 22' 00.12" E	13	8	1	4	6
Z. pallidus	Kimberley (KIM)	Savanna	28° 44' 41.83" S, 24° 45' 29.23" E	9	9	9	9	7

Z. pallidus	Kuruman (KUR)	Savanna	27° 27' 53.30" S, 23° 26' 10.04" E	2	2	2	2	2
Z. pallidus	Lindley (LIN)	Grassland	27° 57' 32.86" S, 27° 56' 39.77" E	3	3	3	3	3
Z. pallidus	Nova Vita (NV)	Nama-Karoo	32° 33' 27.06" S, 21° 25' 58.62" E	1	1	1	1	1
Z. pallidus	Ottoshoop (OH)	Savanna	25° 43' 48.49" S, 25° 58' 28.02" E	1	1	1	1	1
Z. pallidus	Rooiport (RP)	Savanna	28° 41' 45.53" S, 24° 04' 20.94" E	3	1	-	5	4
Z. pallidus	Rouxville (RV)	Grassland	30° 24' 46.94" S, 26° 49' 57.00" E	1	1	1	1	2
Z. pallidus	Sandveld NR (SNR)	Savanna	27° 40' 34.42" S, 25° 41' 00.74" E	13	13	4	11	13
Z. pallidus	Taba Nchu (TN)	Grassland	29° 16' 15.13" S, 26° 54' 22.41" E	2	1	2	2	2
Z. pallidus	Vanderbijlpark (VBP)	Grassland	26° 42' 59.99" S, 27° 52' 00.00" E	5	6	2	7	6
Z. pallidus	Vioolsdrift (VD)	Succulent Karoo	28° 41' 58.69" S, 17° 31' 50.12" E	27	10	7	40	37
Z. senegalensis	Nyika (NKA)	Forest	10° 34' 12.00" S, 33° 51' 00.00" E	6	5	6	6	6
Z. senegalensis	Zomba (ZOM)	Forest	15° 20' 46.71" S, 35° 17' 41.50" E	4	4	4	4	4
Z. virens	Clarens (CLR)	Grassland	28° 31' 05.78" S, 28° 25' 18.95" E	2	1	2	2	1
Z. virens	Eshowe (ESH)	Savanna	28° 53' 44.46" S, 31° 28' 08.55" E	26	15	4	26	28
Z. virens	Harrismith (HRS)	Grassland	28° 16' 46.50" S, 29° 07' 13.57" E	16	11	11	14	17
Z. virens	Jamestown (JT)	Grassland	31° 06' 28.85" S, 26° 49' 10.27" E	5	3	1	5	6
Z. virens	Johannesburg (JHB)	Grassland	26° 13' 29.87" S, 28° 02' 23.06" E	-	-	-	2	2
Z. virens	Kasouga (KSG)	Thicket	33° 39' 35.88" S, 26° 46' 35.78" E	5	5	5	5	5
Z. virens	Komatiepoort (KMP)	Savanna	25° 32' 00.58" S, 31° 41' 12.38" E	4	3	3	4	5
Z. virens	Lindley (LIN)	Grassland	27° 57' 32.86" S, 27° 56' 39.77" E	2	2	1	2	2
Z. virens	Louis Trichard (LT)	Savanna	23° 00' 55.67" S, 29° 36' 00.92" E	13	11	5	12	14
Z. virens	Magoebaskloof (MGK)	Forest	23° 51' 46.13" S, 30° 02' 45.02" E	9	8	4	14	12
Z. virens	Morgan Bay (MB)	Thicket	32° 42' 53.91" S, 28° 27' 10.70" E	10	10	10	8	10
Z. virens	Ngulube (NGU)	Savanna	23° 55' 16.82" S, 30° 51' 15.80" E	1	1	1	1	1
Z. virens	Tarkastad (TKS)	Grassland	32° 00' 52.53" S, 26° 16' 07.88" E	2	2	1	1	2
Z. virens	Vanderbijlpark (VBP)	Grassland	26° 42' 59.99" S, 27° 52' 00.00" E	6	6	2	7	6
Z. virens	Wakkerstroom (WAK)	Grassland	27° 22' 00.26" S, 30° 08' 37.18" E	4	6	2	6	7

Table 5.2: The number of polymorphic sites (P), the number of haplotypes (H), haplotypic diversity (Hd ± sd), nucleotide diversity (π), and Watterson's theta (θ) for each southern African *Zosterops* taxon at each of the five molecular data sets.

		P	H	Hd	π	θ
ATP6						
	Z. pallidus	43	19	0.832 (± 0.033)	0.0066	0.0019
	Z. capensis	67	46	0.927 (± 0.014)	0.0107	0.0175
	Z. senegalensis	7	4	0.644 (± 0.152)	0.0033	0.0035
	Z. virens	70	51	0.933 (± 0.017)	0.0092	0.0188
CHD1						
	Z. pallidus	5	6	0.489 (± 0.047)	0.0012	0.0020
	Z. capensis	13	15	0.564 (± 0.035)	0.0016	0.0046
	Z. senegalensis	20	11	0.856 (± 0.079)	0.0083	0.0122
	Z. virens	20	20	0.658 (± 0.036)	0.0022	0.0081
MUSK						
	Z. pallidus	7	7	0.487 (± 0.062)	0.0013	0.0023
	Z. capensis	8	8	0.675 (± 0.024)	0.0019	0.0024
	Z. senegalensis	8	8	0.889 (± 0.036)	0.0031	0.0038
	Z. virens	10	8	0.592 (± 0.053)	0.0018	0.0033
GAPDH						
	Z. pallidus	8	9	0.699 (± 0.020)	0.0037	0.0042
	Z. capensis	13	14	0.771 (± 0.015)	0.0041	0.0064
	Z. senegalensis	6	6	0.721 (± 0.089)	0.0034	0.0051
	Z. virens	14	16	0.727 (± 0.022)	0.0035	0.0072
TGFβ2						
	Z. pallidus	21	12	0.257 (± 0.042)	0.0019	0.0057
	Z. capensis	22	14	0.480 (± 0.027)	0.0028	0.0056
	Z. senegalensis	27	13	0.905 (± 0.054)	0.0127	0.0121
	Z. virens	25	19	0.562 (± 0.039)	0.0040	0.0066

Table 5.3: A summary table of results from population expansion (using Fu's *Fs* test & Ramos-Onsins and Rozas R^2 statistic) and isolation-by-distance analysis for each *Zosterops* taxa and each Biome of South Africa. Isolation-by-distance analyses could not be done on the Succulent Karoo as only two localities fell within this Biome. + represent evidence ($P < 0.01$) for an analysis, while – represents no evidence for an analysis.

	Z. capensis	*Z. virens*	*Z. pallidus*	*Z. senegalensis*	Forest	Fynbos	Grassland	Nama-Karoo	Savanna	Succulent Karoo	Thicket
ATP6											
Population expansion	+	+	-	-	-	-	-	-	-	-	-
Isolation-by-distance	-	-	-	-	-	-	-	-	-	-	-
CHD1											
Population expansion	+	+	-	-	+	-	+	+	+	-	-
Isolation-by-distance	-	-	-	-	-	-	-	-	-	-	+
MUSK											
Population expansion	-	-	-	-	+	-	-	-	-	-	-
Isolation-by-distance	-	-	-	-	-	-	-	-	-	-	-
GAPDH											
Population expansion	+	+	-	+	+	-	-	-	+	-	+
Isolation-by-distance	-	-	-	-	-	-	-	-	-	-	-
TGFβ2											
Population expansion	-	-	-	-	+	-	-	-	-	+	+
Isolation-by-distance	-	-	-	-	-	-	-	-	-	-	-

Table 5.4: Parameter estimates (θ, M and T), divergence times (years) and time since most recent common ancestor (years) of the pairwise comparisons of *Z. capensis, Z. pallidus, Z. virens* and *Z. senegalensis* generated by a 10 million generation run in MDIV (HKY model) for each locus. Priors were set as M_{max} = 10 for ATP6, MUSK and TGFβ2, and M_{max} = 20 for GAPDH and CHDZ. T_{max} = 25 was set for all loci.

Species pair	Locus	θ	M	T	t div	Tmrca
capensis/*virens*	ATP6	17.63 (16.65 - 18.6)	3.38 (3.13 - 3.63)	0.35	174 560	1 351 213
	CHD1	3.91 (2.94 - 4.89)	12.68 (12.43 - 12.93)	0.2	168 538	1 207 193
	MUSK	1.42 (0.45 - 2.39)	6.86 (6.61 - 7.11)	0.15	57 352	1 033 928
	GAPDH	2.84 (1.87 - 3.82)	19.96 (19.71 - 20.21)	5.12	143 277	2 821 377
	TGFβ2	3.8 (2.83 - 4.78)	7.74 (7.49 - 7.99)	0.2	168 147	4 010 743
capensis/*pallidus*	ATP6	10.98 (10.27 - 11.69)	0.28 (0.03 - 0.53)	4.6	1 428 878	1 618 454
	CHD1	1.91 (1.75 - 2.08)	6.56 (6.31 - 6.81)	0.1	41 193	801 455
	MUSK	0.99 (0.87 - 1.12)	0.7 (0.45 - 0.95)	2.35	629 688	1 040 829
	GAPDH	2.3 (2.14 - 2.46)	1.84 (1.59 - 2.09)	1.45	1 406 502	3 894 060
	TGFβ2	2.81 (2.53 - 3.09)	1.14 (0.89 - 1.39)	13.85	6 617 613	4 192 745
virens/*pallidus*	ATP6	14.49 (13.52 - 15.47)	1.14 (0.89 - 1.39)	3.55	1 455 760	1 382 650
	CHD1	3.17 (2.19 - 4.14)	3.22 (2.97 - 3.47)	0.1	68 264	1 140 342
	MUSK	1.49 (0.52 - 2.47)	0.46 (0.21 - 0.71)	1.9	764 149	1 236 715
	GAPDH	3.02 (2.04 - 3.99)	1.14 (0.89 - 1.39)	2.55	3 249 122	4 992 112
	TGFβ2	3.24 (2.26 - 4.21)	1.32 (1.07 - 1.57)	6.2	4 435 508	4 155 220
virens/*senegalensis*	ATP6	21.92 (20.99 - 22.83)	0.16 (0 - 0.41)	0.5	310 072	1 478 697
	CHD1	6.81 (6.35 - 7.27)	0.32(0 - 1.05)	0.64	939 714	2 338 646
	MUSK	2.47 (2.21 - 2.73)	0.22 (0 - 0.47)	0.5	332 806	1 297 073
	GAPDH	5.19 (5 - 5.38)	1.64 (1.13 - 2.15)	0.16	350 429	6 097 996
	TGFβ2	5.78 (5.16 - 6.40)	0.5 (0.25 - 0.75)	2.65	3 386 745	4 099 167

Figure legends

Figure 5.1: a) The biomes of South Africa showing the sampled localities of *Z. capensis* (blue), *Z. pallidus* (red), *Z. senegalensis* (yellow) and *Z. virens* (green). Purple circles are localities of contact. Full names and geographic co-ordinates of abbreviated locality names are shown in Table 5.1; b) the distributional ranges on *Z. capensis* (blue), *Z. pallidus* (red), *Z. senegalensis* (yellow) and *Z. virens* (green) in southern Africa. Full names of abbreviated locality names are shown in Table 5.1.

Figure 5.2: Parsimony networks of mitochondrial ATP6 of all southern African *Zosterops* taxa. Two networks emerged, not connected by a 95% connection limit. Blue = *Z. capensis*, Orange = *Z. pallidus*, Yellow = *Z. senegalensis*, Green = *Z. virens*, Purple = individuals with a genotype/phenotype mismatch. The WC haplotype, and the blue haplotypes connected to it, represent *Z. capensis* individuals collected from Western Cape localities. Open circles represent unsampled alleles.

Figure 5.3: Parsimony networks of sex-linked loci CHDZ and MUSK, and autosomal loci GAPDH and TGFβ2 of all southern African *Zosterops* taxa. All parsimony networks were performed on phased data. Blue = *Z. capensis*, Orange = *Z. pallidus*, Yellow = *Z. senegalensis*, Green = *Z. virens*, Purple = individuals with a genotype/phenotype mismatch. Open circles represent unsampled alleles.

Figure 5.4: AMOVA bar graphs representing differences in among-group (Φ_{CT}), among populations within group, and within population variation among four grouping criteria: A) three phylogroups, B) four morphogroups, C) four phylogroups and D) Biome groups (see text for details). Comparisons were made for all loci. Bars marked with * show among group variation (Φ_{CT}) that is significant ($P < 0.001$).

Figure 5.5: The number of populations estimated from STRUCTURE: a) The mean estimate of the probabilities of the data (LnPr L(*K*)) over 10 replicated runs for each value of *K*, b) Values of Δ*K* (calculated as $\Delta K = m|L''(K)|/s[L(K)]$; Evanno *et al*. 2005) plotted against values of *K*.

Figure 5.6: Structure plot based on the combined nuclear data set (autosomal: TGF-B2 and GAPDH, and sex-linked: CHD and MUSK) of 361 southern African *Zosterops* individuals.

The groupings at $K = 2$ to $K = 5$ of the four phenotypic Zosterops taxa are shown (*pallidus, capensis, virens* and *senegalensis*). Each individual is represented by horizontal lines divided into coloured segments that represent membership to each cluster.

Figure 5.7: Posterior probability distribution estimates of effective population size, for: a) *Z. capensis* and *Z. virens*, b) *Z. pallidus* and *Z. virens* and c) *Z. virens* and *Z. senegalensis*. Also shown are the posterior probability distributions of the *M* parameters in each direction between: d) *Z. capensis* and *Z. virens*, e) *Z. pallidus* and *Z. virens* and f) *Z. virens* and *Z. senegalensis*. The posterior probability distribution of the *t* parameter between *Z. capensis* and *Z. virens*, between *Z. pallidus* and *Z. virens*, and between *Z. virens* and *Z. senegalensis* is also presented (g).

Figure 5.8: A multi-dimensional scaling analysis of a) 19 environmental bioclimatic variables for each of the *Zosterops* collecting localities. Green = *Z. virens*, blue = *Z. capensis*, red = *Z. pallidus*, yellow = *Z. senegalensis*, black = localities with multiple taxa. A magnified view (b) reveals the position of localities where multiple taxa were collected: Aliwal North (AN), Rouxville (RV) and Taba Nchu (TN) (*Z. pallidus* and *Z. capensis*); Lindley (LIN) and Vanderbijlpark (VBP) (*Z. pallidus* and *Z. virens*); Clarens (CLR) and Tarkastad (TKS) (*Z. capensis* and *Z. virens*). Full names of abbreviated locality names are shown in Table 5.1.

Fig. 5.1a

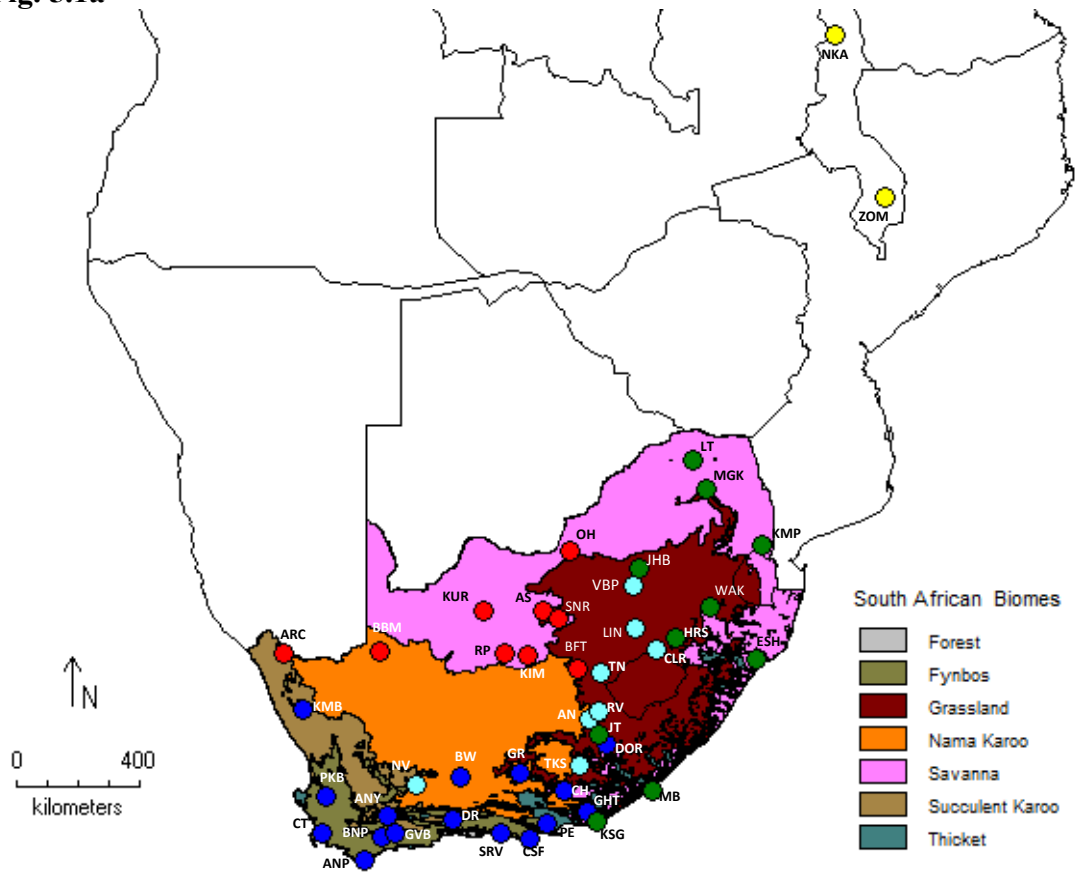


Fig. 5.1b

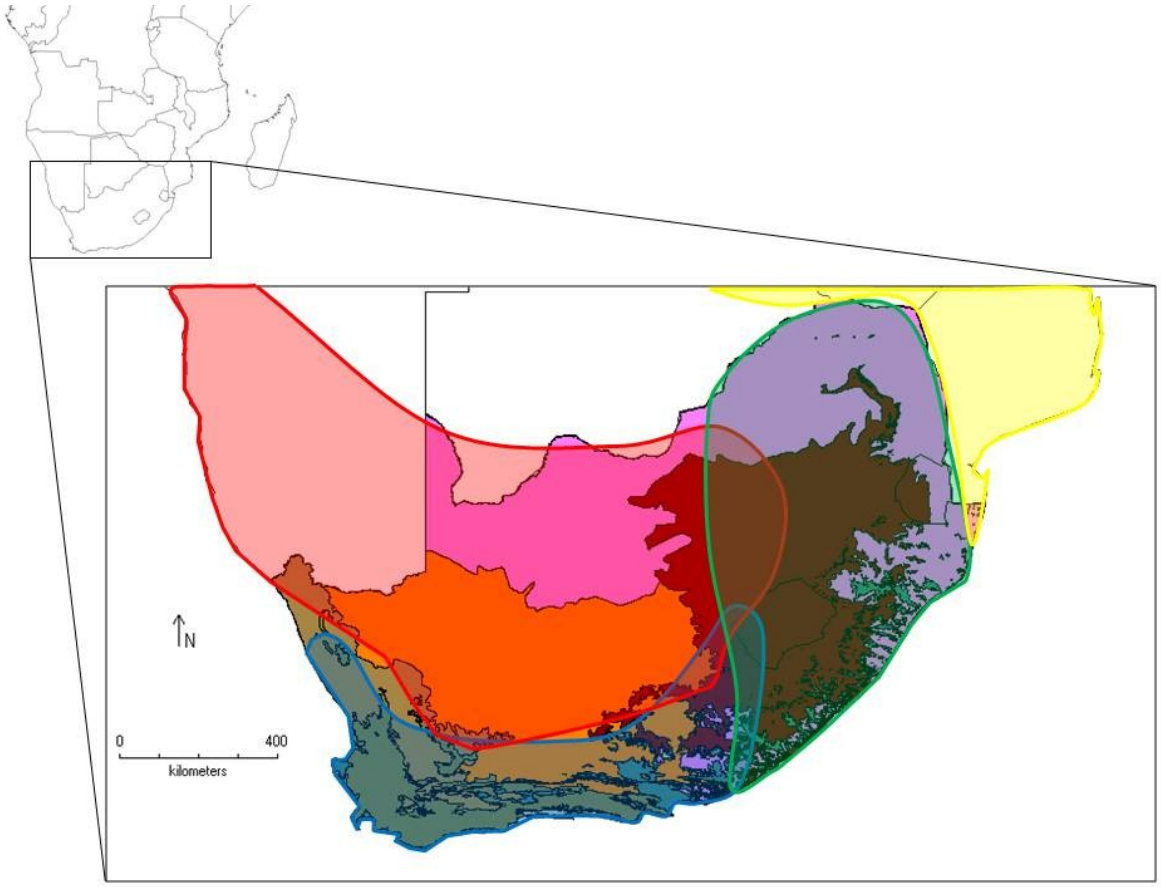
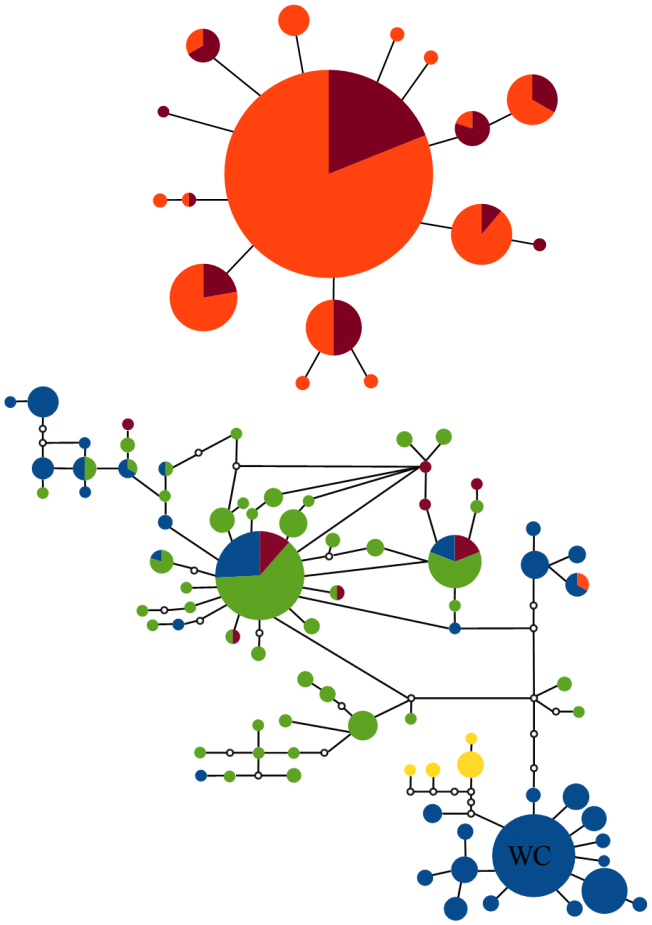


Fig. 5.2



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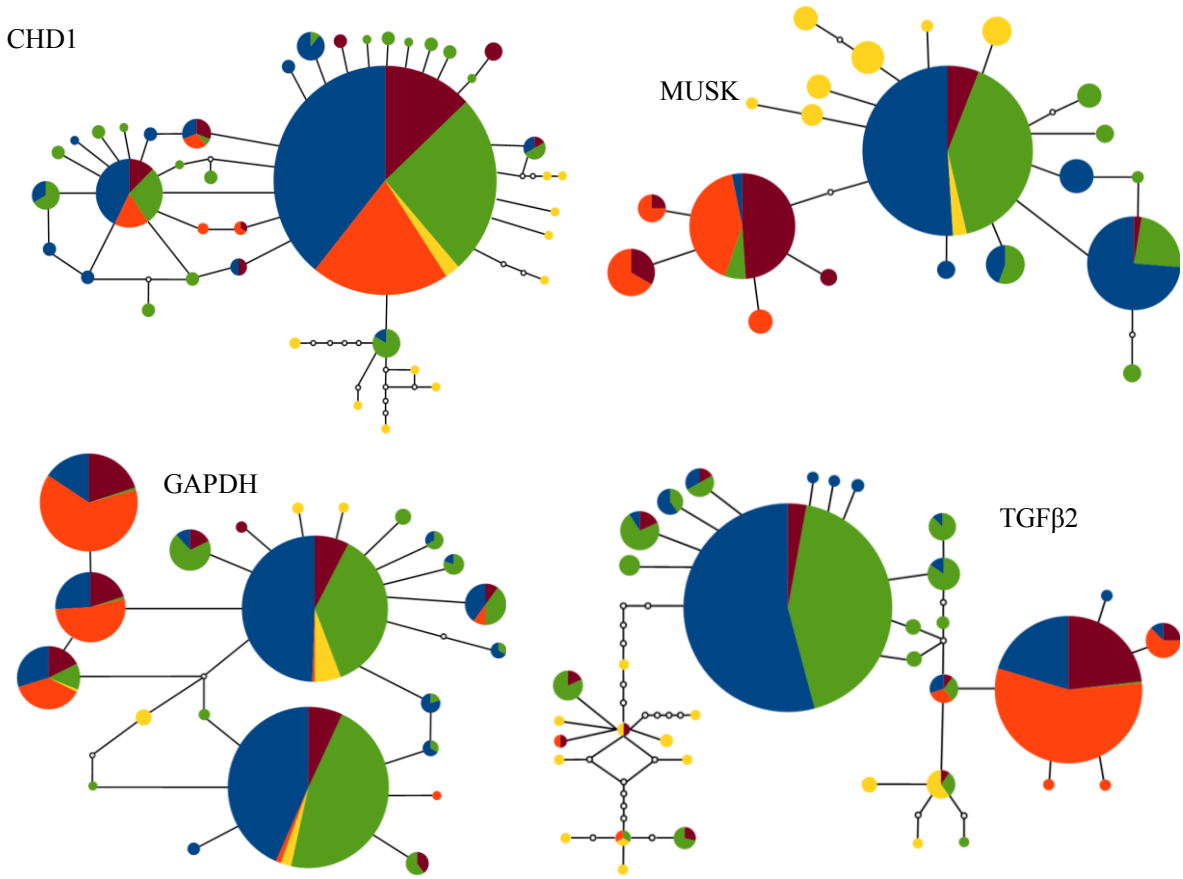

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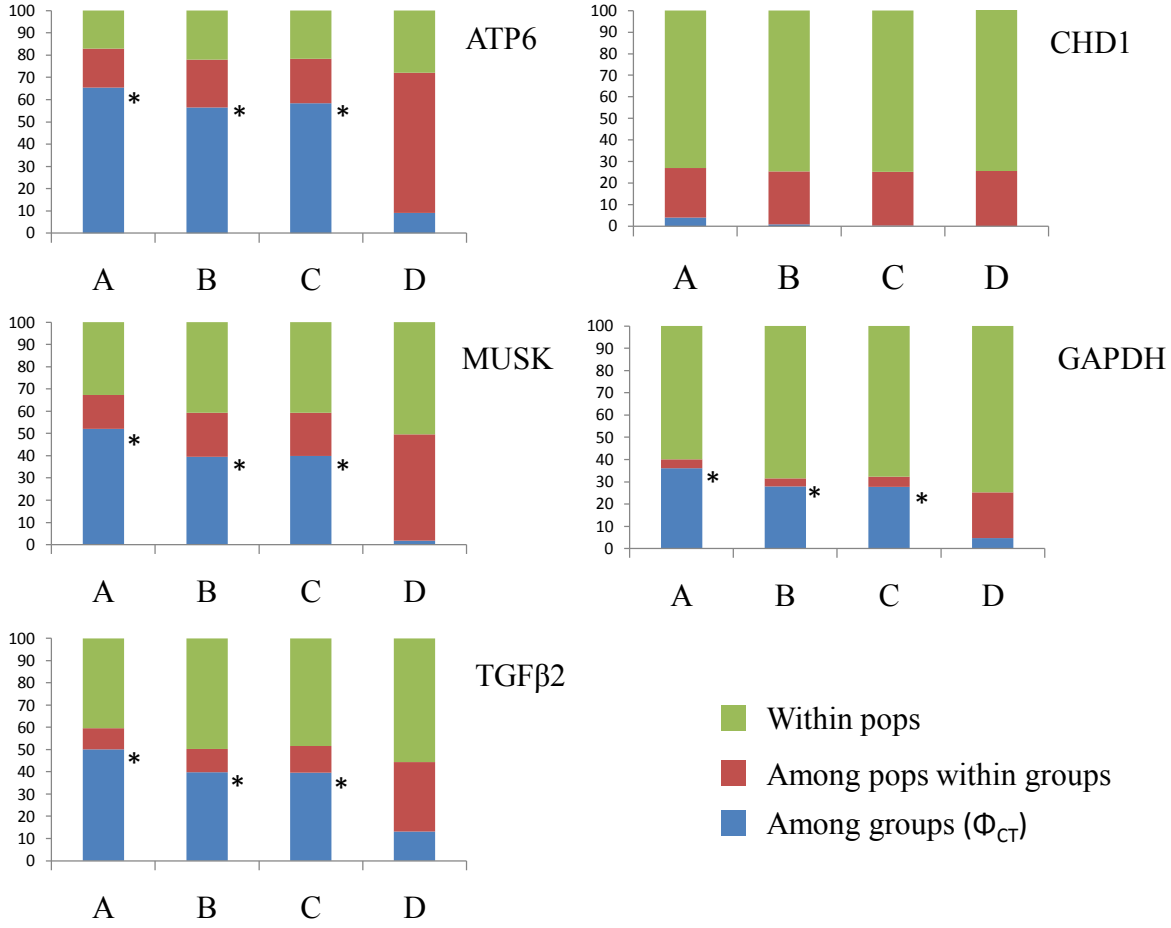

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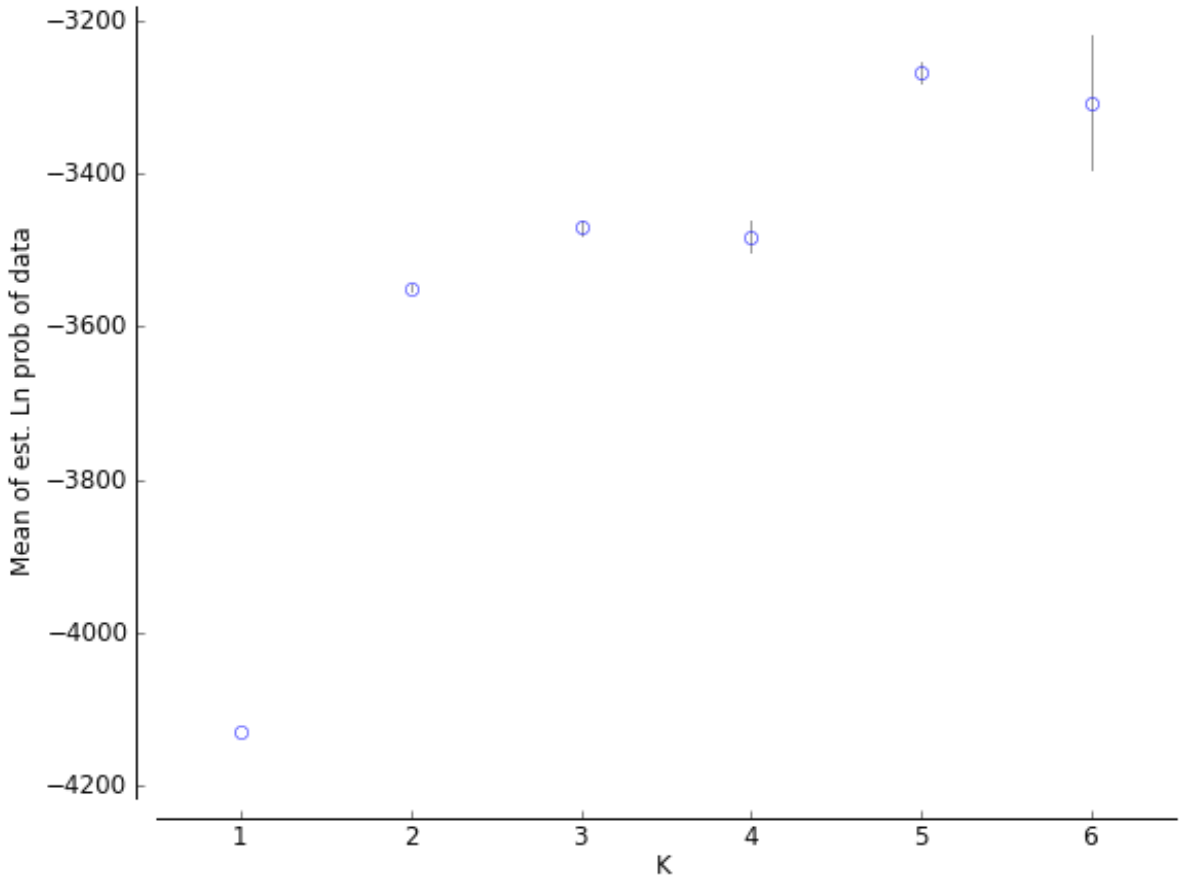
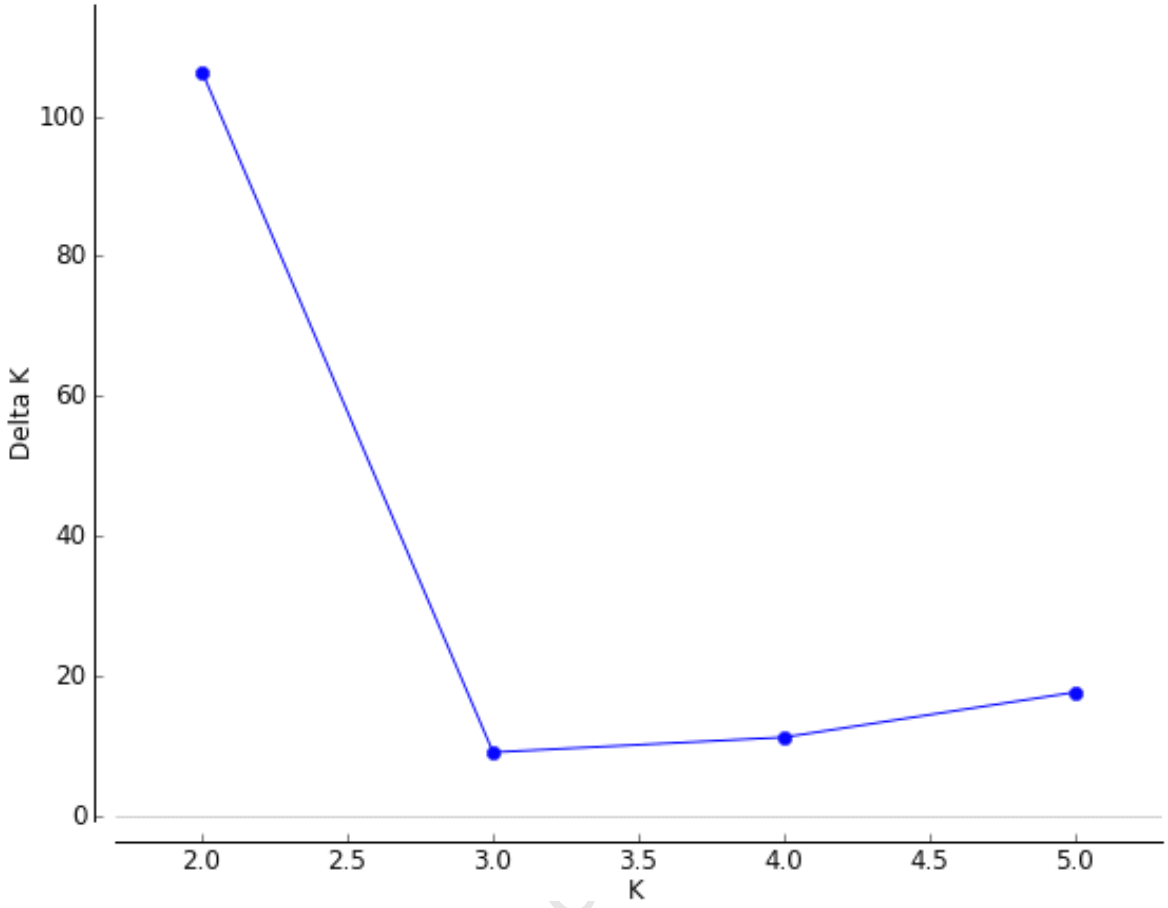


Fig. 5.5b



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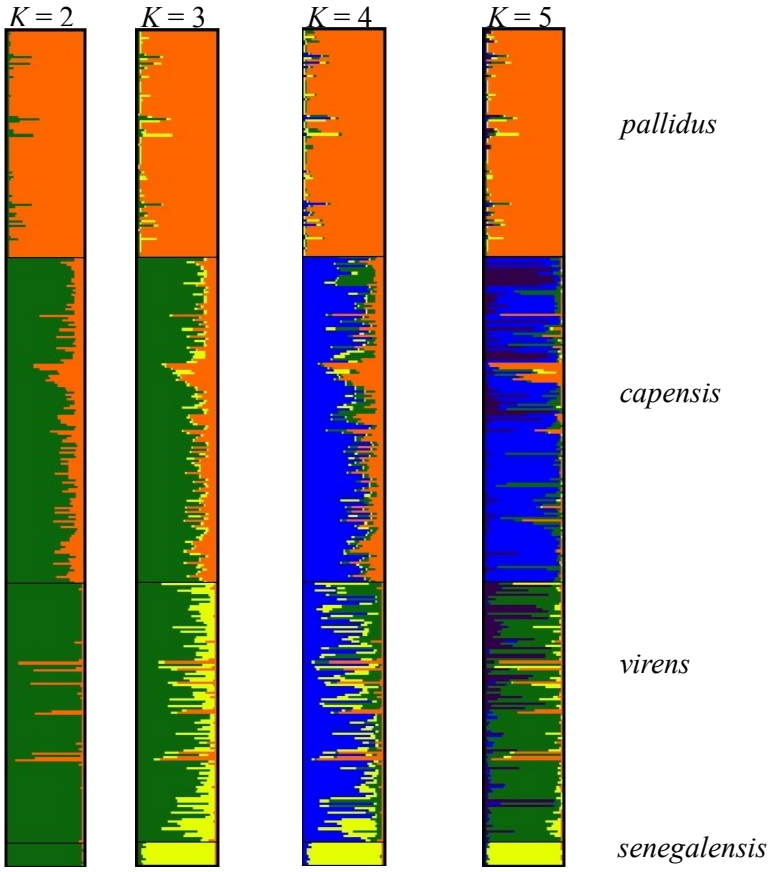
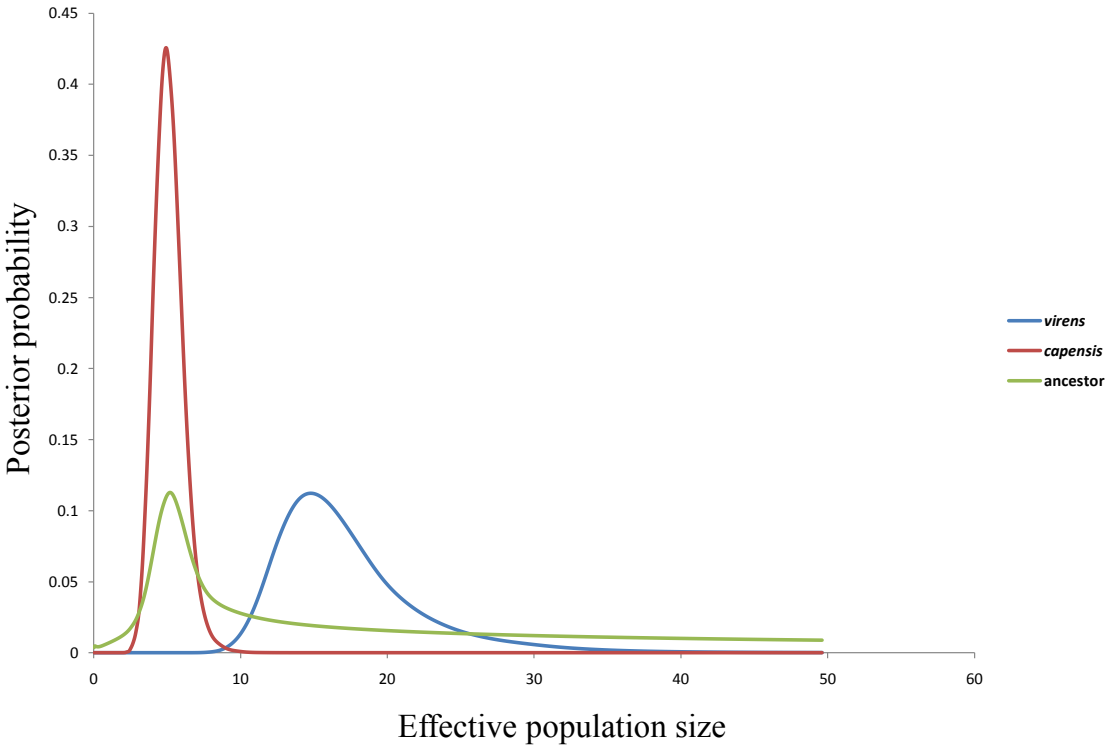


Fig. 5.7a



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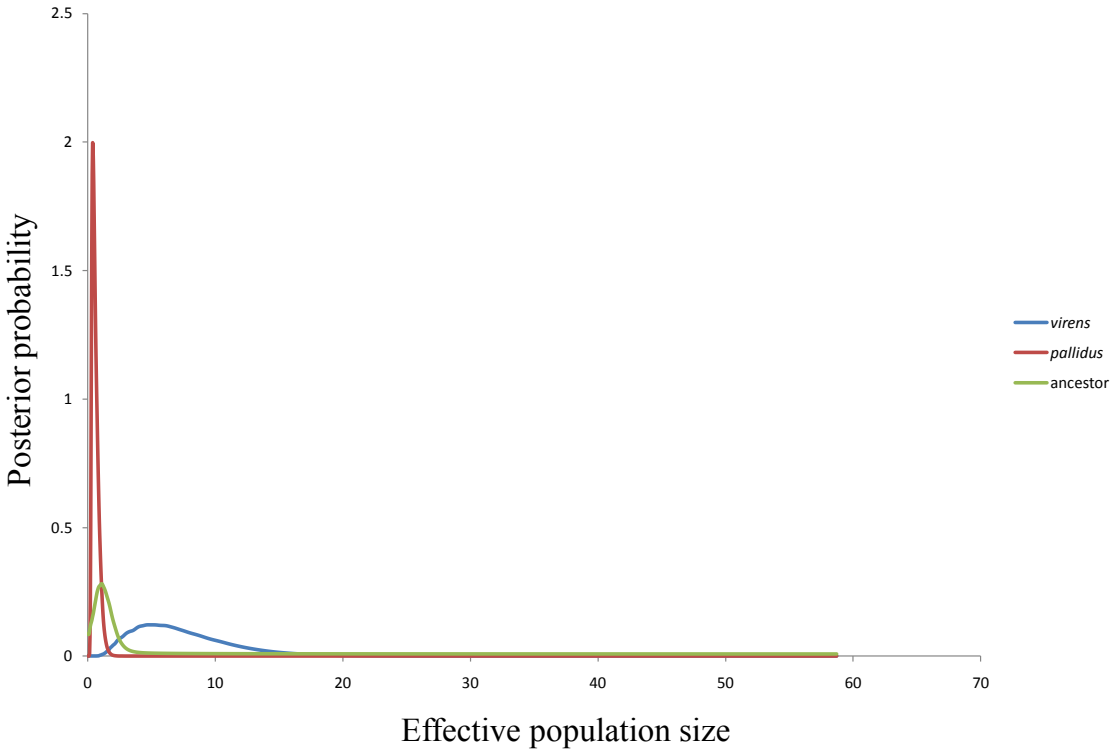

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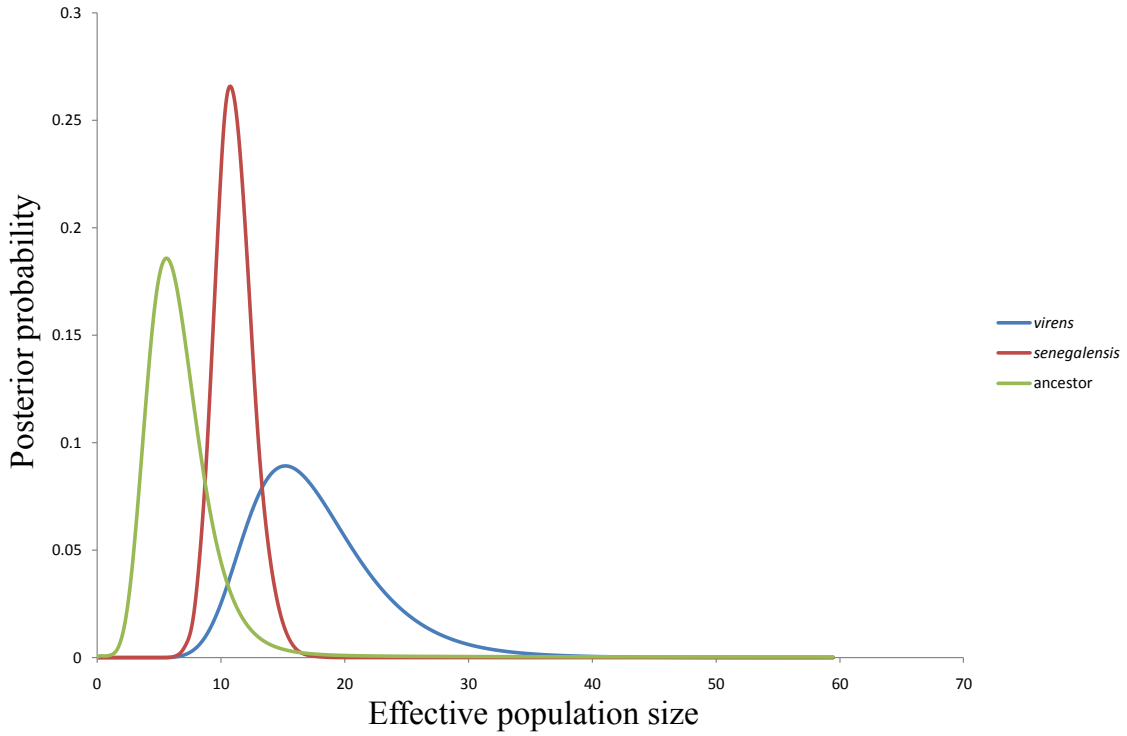

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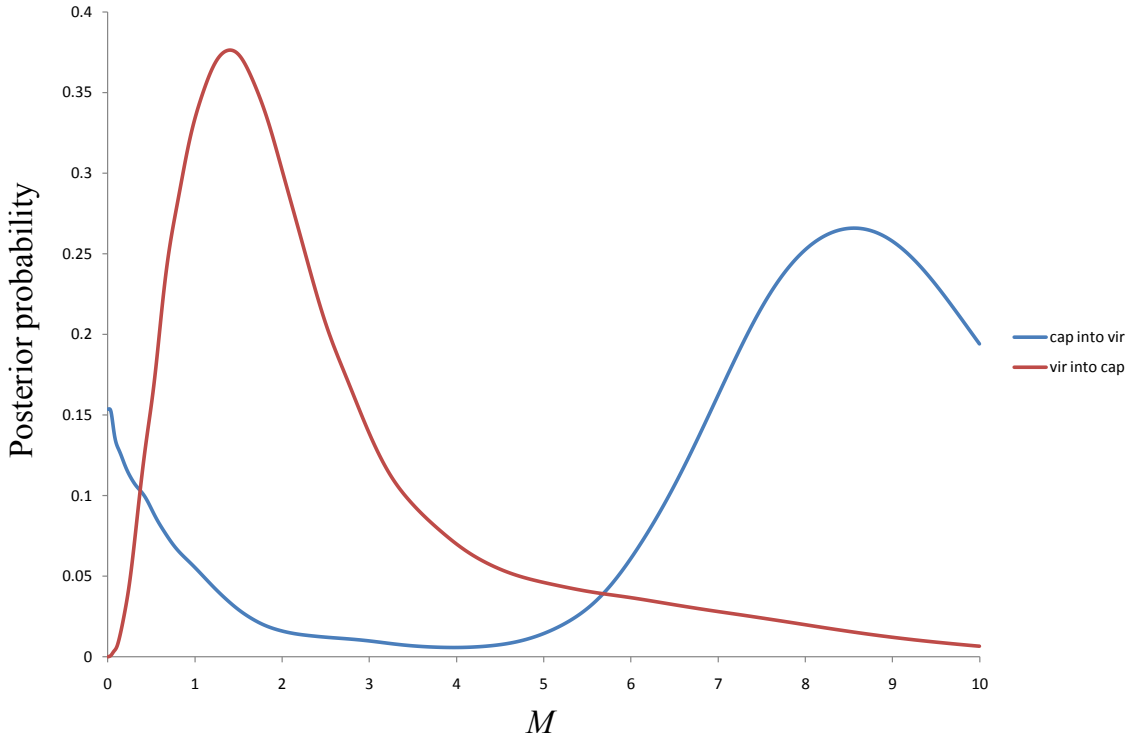

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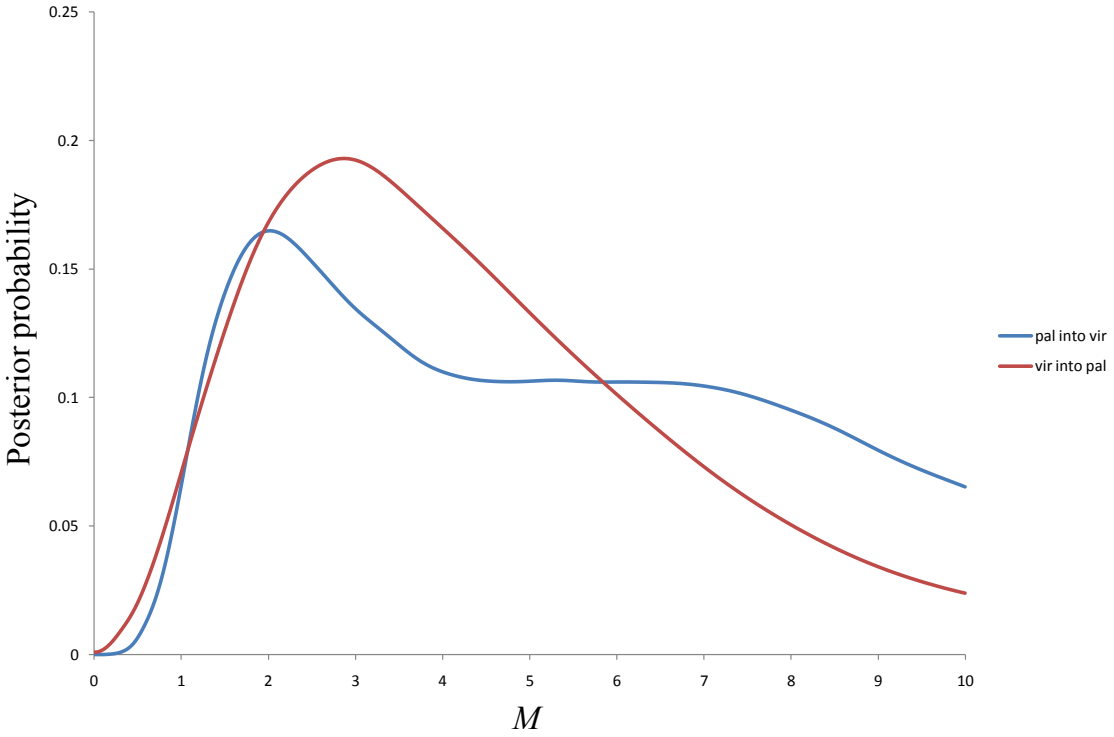


Fig. 5.7f

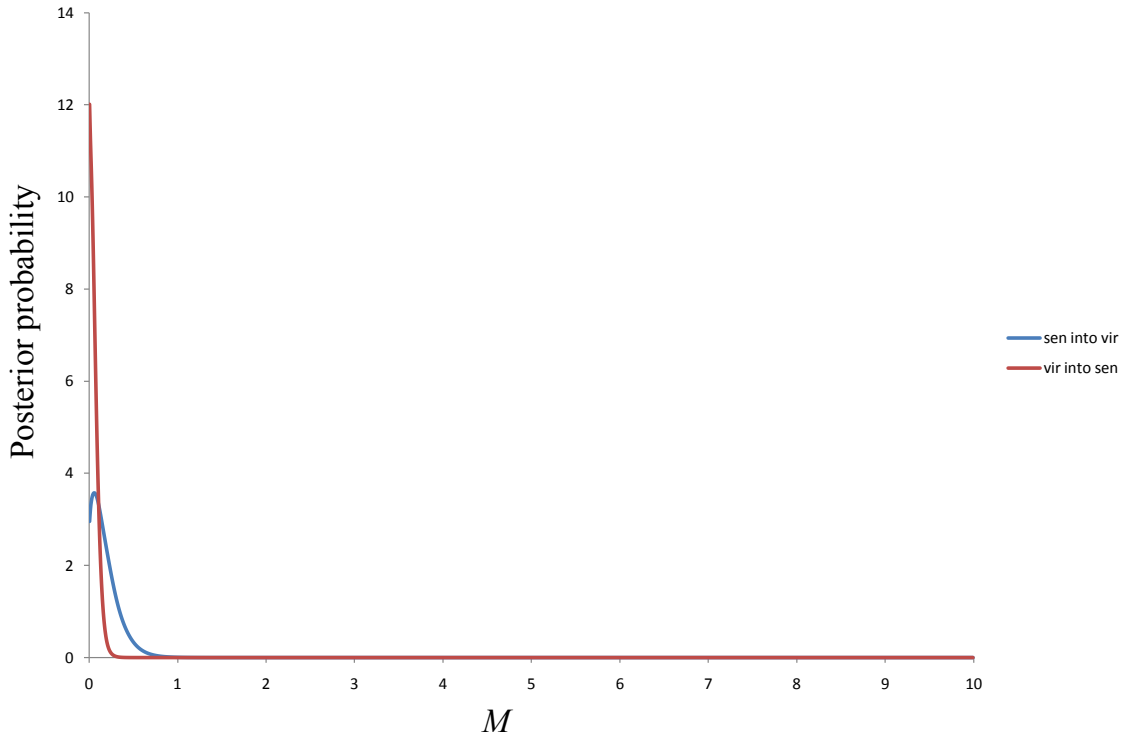


Fig. 5.7g

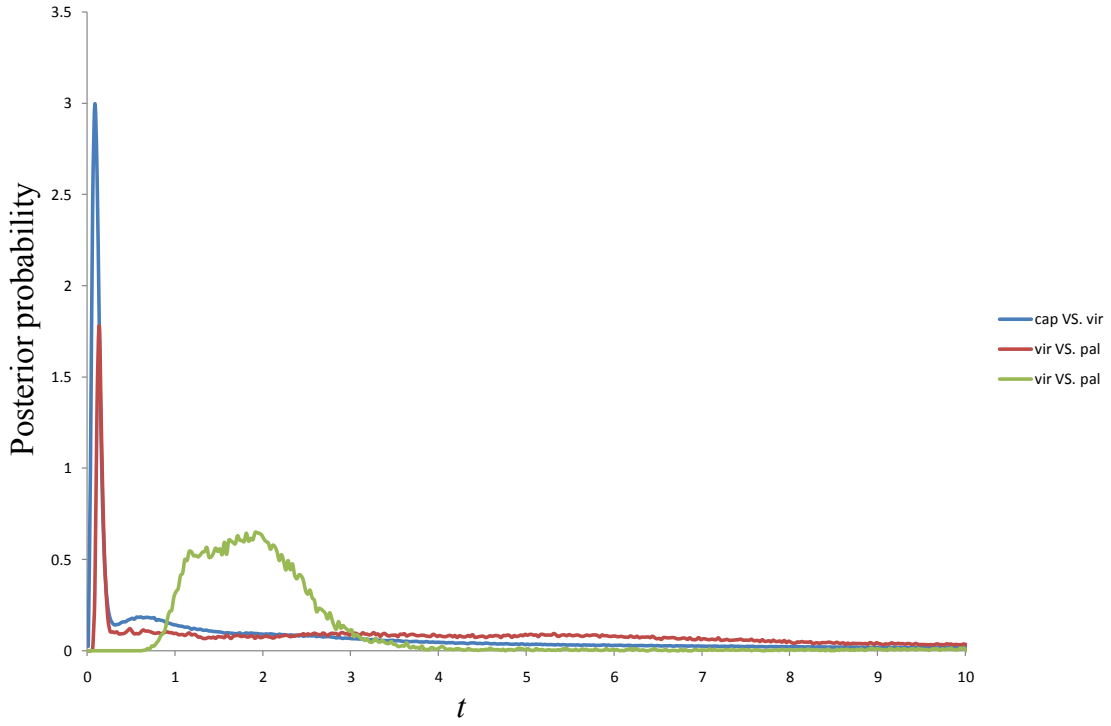


Fig. 5.8a

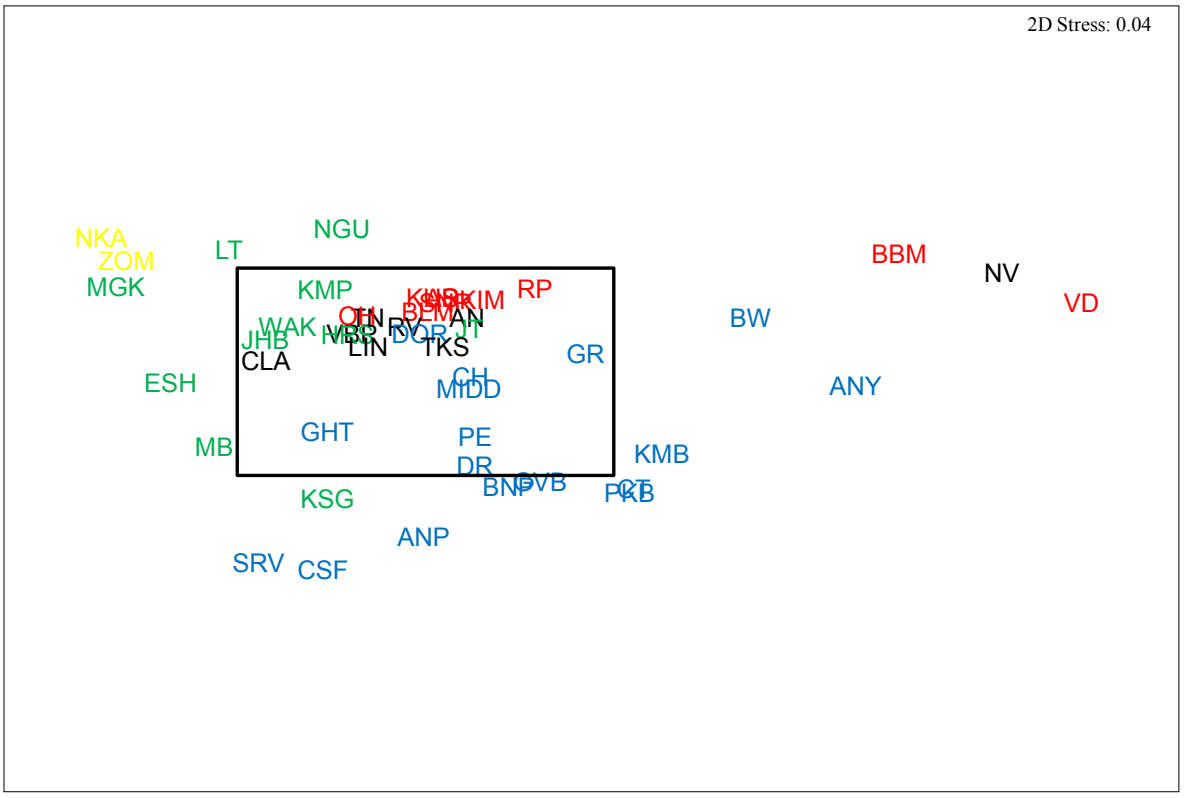
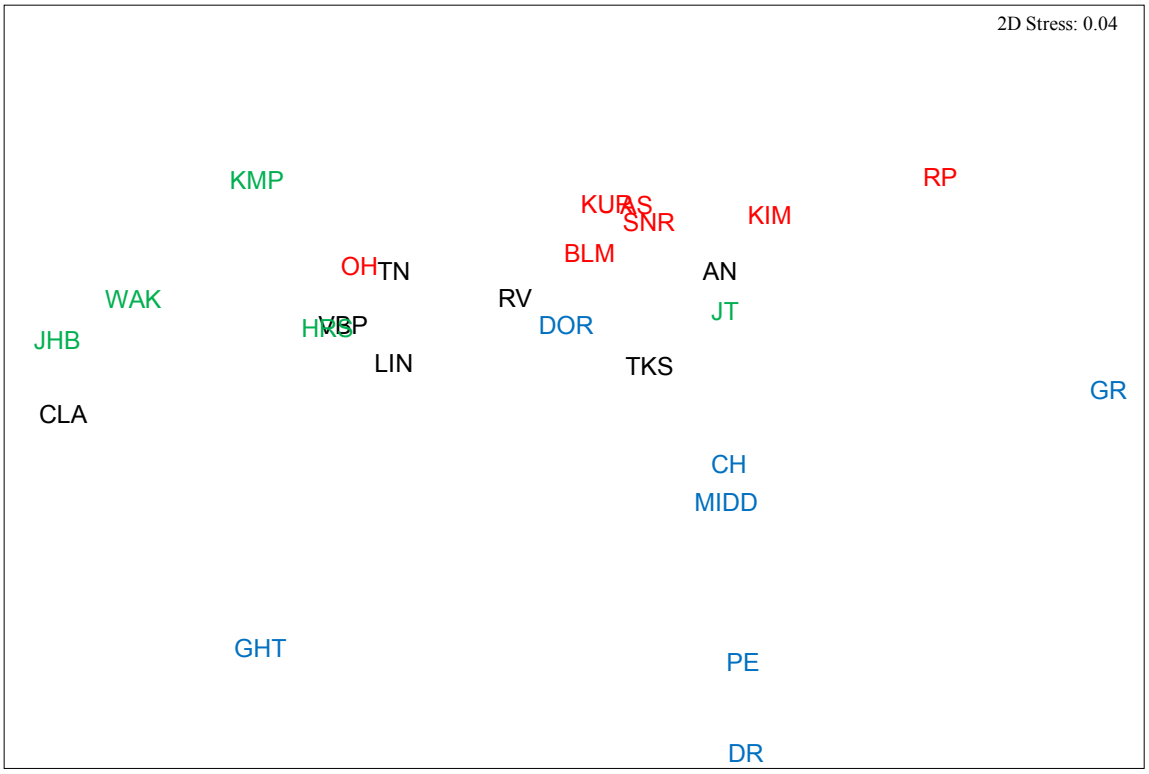


Fig. 5.8b



Chapter 6

Conclusions and future prospects

The process of identifying discrete biodiversity units and the evolutionary forces underpinning these units is an important step in conservation biology (Funk *et al.* 2002) and biology in general. Systematic research can be used to identify historical evolutionarily significant units that need to be preserved, since lineages lost are unlikely to be recovered (Moritz 2002). Another equally important aspect of systematic research is to gain an understanding of the evolutionary processes driving observed diversity. These results can then be used to highlight areas of conservation priority that not only conserve lineage diversity, but also the mechanisms which lead to its formation (Desmet *et al*. 2002).

With the introduction, and subsequent dominance of molecular methods in systematic research, the 1990s and early 2000s saw a decrease in the use of traditional systematic data such as morphological character sets. A call for a more complete multifaceted approach towards systematic studies (Hillis 1987, Crowe 1999, Will and Rubinoff 2004, Dayrat 2005, Tobias *et al*. 2010a) has seen a recent trend of incorporating multiple character data sets that contribute more thoroughly to the establishment of biodiversity estimates (see Fjeldså *et al.* 2006, Alström *et al.* 2008, Phillimore *et al.* 2008, Rheindt *et al.* 2008, García *et al.* 2008, Kearns *et al.* 2009, Uy *et al.* 2009, Cadena and Cuervo 2010). The sole reliance on molecular markers (either a single marker or multiple markers) for species delimitation can provide issues of uncertainty, as in the case of gene trees not necessarily being the same as species trees (Doyle 1992, Moore 1995) through processes such as homoplasy (Edwards *et al.* 1991, Klicka *et al.* 2000, Barker *et al.* 2002, Engstrom *et al*. 2004; but see Källersjö *et al.* 1999), recombination (Posada and Crandall 2002, Ruths and Nakhleh 2005) and incomplete lineage sorting (Avise *et al.* 1983, Maddison 1997, Funk and Omland 2003, Rosenberg 2003).

Although these problems can be overcome with the use of various analytical and sampling approaches (Maddison and Knowles 2006, Knowles and Carstens 2007), the complexity of biological species and the boundaries that delimit these species necessitate information from multiple character sets in order to make informed, and more importantly, correct biodiversity estimates (Dayrat 2005). The use of several sources of systematic characters also provides the opportunity to determine the various (e.g. sexual selection, local adaptation, drift, competition) processes that may be responsible for the formation of the biodiversity observed.

The southern African *Zosterops* (white-eyes) species complex represents a group of birds where the systematic relationships among taxa has been debated, but has remained unresolved for many decades. From all classifications of southern African *Zosterops* (Gill 1936, Roberts 1942, Moreau 1957, Skead 1967, Clancey 1967, Hockey *et al.* 2005, van Balen 2008), multiple taxa (both species and subspecies) have been named and described (Chapter 1, Table 1.1). The earlier classifications (Gill 1936, Roberts 1942, Moreau 1957) centred on underpart plumage colouration, however later classifications (Clancey 1967, van Balen 2008) have focused on interbreeding and lumped taxa into a single species (i.e. *Z. pallidus*). White-eyes of the region can be broadly split into four underpart plumage groups: grey, green, pale (with cinnamon flanks) and yellow, however the true systematic relationship among these birds has yet to be established. The purpose of this study has been to incorporate multiple avenues of evidence in order to establish the taxonomic relationships among southern African *Zosterops* and to determine the evolutionary processes that have driven the observed biodiversity in the region.

Establishing hypotheses of *Zosterops* biodiversity begun by determining the number of morphospecies (*sensu* Cain 1954) using the morphometric and plumage characters available to previous authors, and by using modern methodologies and techniques to analyze

the data. The resultant biodiversity hypothesis is then tested using vocal (song and contact call) and molecular (mitochondrial and nuclear DNA) data to reject or accept the hypothesis.

6.1. Morphology

Even given the numerous drawbacks in using morphology as a the sole taxonomic tool for taxon delimitation (Mayr *et al.* 1953, Wilson and Brown 1953, Cain 1954, Zink 2004), morphometric and plumage characters can be used to establish whether there is the presence of discernable Evolutionarily Significant Units (ESU) or eco-geographic variants with respect to the environmental heterogeneity represented by the diverse biomes of southern Africa. Necessarily

Standard avian morphometric measures (bill-, tarsus-, wing- and tail-length) and two measures of underpart plumage colouration were obtained from 558 museum and freshly collected specimens that covered the range of all previously described southern African *Zosterops* taxa. The first underpart plumage measure estimated percentage colour (either grey, green, cinnamon, yellow or white) from digital photographs of the specimens, while the second used Adobe® Photoshop® 7.0.1 (Adobe Systems, Inc., San Jose, CA, USA) to obtain quantitative colour scores from standardized digital photographs. Uni- and multi-variate statistical analyses were conducted on each data set separately, and as a combined data set.

Morphometric measures alone proved to be unsatisfactory as taxonomic indicators, whereas both underpart plumage colour data sets were taxonomically informative and showed the presence of four ESU. A combination of the morphometric and percentage underpart colouration data set proved to be the best discriminatory characters, allowing the more technical and time consuming quantitative colour method to not be required as a tool to distinguish *Zosterops* taxa taxonomy.

The lack of clinal variation among the four morphological ESU indicates that these taxa appear to be on their own evolutionary trajectories, and that they do not represent eco-geographic variants with gradual changes in morphological characters between populations. The high diagnosability amongst them suggests that the four *Zosterops* ESU can be recognized as good morphological species: Grey Cape White-eye *Z. capensis*, Orange River White-eye *Z. pallidus*, African Yellow White-eye *Z. senegalensis* and Green Cape White-eye *Z. virens*. The validity of these species is tested further with the addition of other lines of systematic evidence: vocalizations, mitochondrial and nuclear DNA.

6.2. Vocalizations

Whereas morphological approaches to taxonomy and phylogenetics provide indications of possible biodiversity, numerous issues such as sexual dimorphism, morphological variation among age classes, plumage or size polymorphisms and local variation within and among allopatric populations/taxa (Mayr *et al.* 1953, Wilson and Brown 1953, Cain 1954, Zink 2004) make the sole reliance on this methodology difficult. As well as being an additional source of taxonomic information, vocal characters offer increased systematic power to avian classifications (Alström and Ranft 2003). Bird songs have many functions and include mate stimulation and defence of territories (Kroodsma and Byers 1991), whereas bird calls maintain contact between individuals within a population or species (Marler 2004). Bird songs act as a reproductively isolating mechanism (Collins 2004) and should be good-species level taxonomic characters. However, care must be taken when deciding on which vocal characters to use as the complexities of vocal learning (Beecher and Brenowitz 2005) and convergence (McCracken and Sheldon 1997) can distort phylogenetic signal.

Both song and contact call data were used in this study as taxonomic characters for African *Zosterops*. Song recordings from *Z. capensis*, *Z. pallidus*, *Z. senegalensis* and *Z. virens* were used, whereas contact call data for *Z. capensis*, *Z. pallidus*, *Z. virens* and phenotypic hybrids were used. As mentioned above, song learning in Oscine passerines (Beecher and Brenowitz 2005) makes it difficult to determine taxonomic characters from passerine song, resulting in either plasticity (Krebs and Kroodsma 1980, Mundinger 1982), or a range of repertoires or mimicry (Kelley *et al.* 2008). Additionally, local dialects may also provide difficulties in establishing taxonomic charaters. As both cultural learning and local dialects are likely to produce complex variation among geographic populations in southern African *Zosterops*, general characters were chosen for analyses to avoid the effects of plasticity shaping taxonomic inferences (Tobias *et al.* 2010a).

Results from the song data were not congruent with the morphological hypothesis of *Zosterops* diversity. Significant differences are observed for the majority of the song measures, with the songs of *Z. pallidus* being different from the other three taxa. Differences in song have been noted previously (Skead 1967), however this is the first instance where the actual components causing these differences have been quantified. Although the mean frequency per song is higher in the other taxa, the maximum element frequency of *Z. pallidus* songs is higher and also made up of short elements delivered at a high rate. These characteristics give the *Z. pallidus* song a sharper sound than those of the other taxa.

Although *Z. senegalensis* contact calls were not available for analysis, taxonomic conclusions could be drawn from the data as well as provide interesting insights into contact zone dynamics between *Z. pallidus* and both *Z. capensis* and *Z. virens*. *Zosterops pallidus* contact calls are composed of short elements and are delivered at a faster rate than those of the other two taxa, whereas hybrid individuals show contact call characteristics of both parental forms. The intermediacy of hybrid individual contact calls provides insight into the

factors that affect the vocal differences observed. With both physiological and neurological processes being important aspects in vocal production (Marler 2004), syrinx morphology and/or the vocal neurological pathways of hybrid individuals may have to change from that of either of the parental forms in order for these individuals to produce intermediate calls. This process however, would involve the transfer of genes responsible for functions from each parental taxon, and act in a "blending" fashion, a model documented in suboscine birds which do not learn their song. The intermediate nature of hybrid vocalizations found in *Zosterops*, an oscine passerine that learn their song, suggests an interesting genetic role that should be explored further.

Contact calls have been widely thought to be innate and not subject to learning (Lanyon 1960, Hinde 1970), but others have found this not to be necessarily true for some taxonomic groups (see Mundinger 1979 and references therein). Up until now, no evidence for call learning has emerged for Zosteropidae. The emergence of intermediate contact calls in hybrid individuals suggests that learning from pure parental forms is influencing the call production in hybrids. Although only speculative at present, imprinting experiments might provide a clearer understanding on call learning in Zosteropidae.

The vocalization data presented here do not support the *Zosterops* classification presented through morphological analyses. The similarity in song between the Grey Cape White-eye *Z. capensis*, the African Yellow White-eye *Z. senegalensis* and the Green Cape white-eye *Z. virens* suggests either recent divergence of these taxa with insufficient time for vocal divergence, or a similarity of habitat type resulting in song convergence. The difference between the Orange River White-eye *Z. pallidus* vocalizations with the other *Zosterops* taxa is either due to a more ancient split between these taxa resulting in vocalization divergence or due to habitat differences – a more open environment, compared to a closed forest

environment. Further work on the processes, such as sexual selection or stochasticity, affecting *Zosterops* vocalization differences will however need to be conducted.

6.3. Molecular phylogeny

Molecular data are commonly used modern systematic research, irrespective of whether other lines of evidence are used. Calls have been made for the sole use of DNA in species delimitation (Tautz *et al.* 2002, 2003 Herbert *et al.* 2003, Mallet and Willmott 2003). However, traditional taxonomic practices (morphology, behavior, ecology) are still very important components to establishing good species (Sites and Marshall 2004, DeSalle *et al.* 2005, Pruett and Winker 2010, Schlick-Steiner *et al*. 2010). A universal methodology for delimiting molecular species boundaries has yet to be reached (DeSalle *et al.* 2005), however, a wide range of options are available that include tree-based clustering (Sites and Marshall 2004) and coalescent (Knowles and Carstens 2007, Yang and Rannala 2010) analyses.

Five molecular (one mtDNA and four nuDNA) markers were sequenced for multiple individuals from each of the putative white-eye taxa in order to test whether the four species of *Zosterops* hypothesized by morphometric and plumage characters are valid. Initial mtDNA and combined nuDNA Bayesian analyses using 75 individuals revealed hybridization between *Zosterops* taxa in areas of contact. In order to establish the true molecular phylogeny of southern African *Zosterops*, individuals from areas of contact were removed in order to exclude the effects that hybridization may have on the true species phylogeny.

Separate parsimony networks for each locus showed congruent patterns (barring the sex-linked CHD1 locus), with the ancestral placement of *Z. pallidus* to the other southern African taxa. The networks also illustrate the differences between using mtDNA and nuDNA, with the greater structure obtained in the mtDNA ATP6 network. This is due to the greater effective population size and lower mutation rate of the diploid nuDNA loci (Moore 1995,

Hare 2001). A combined nuDNA network agreed with the separate molecular networks. The use of nuclear introns in phylogenetic reconstruction is recent (Friesen *et al.* 1997) and does have positive and negative aspects. Although not encountered during this study, indels, coupled with high rates of variation, can provide difficulties with sequence alignment (Yu *et al.* 2011), whereas paralogy and recombination are issues that can be overcome (Creer 2007). Introns provide increased resolution to phylogenies based on other molecular markers (Sang 2002, Benavides *et al.* 2007, Matthee *et al.* 2007, Dalebout *et al.* 2008) and their rate of evolution, although less than that of mtDNA, is sufficient to provide meaningful taxonomic information (Yu *et al.* 2011). The use of nuclear introns in this study has not been subject to the difficulties experienced in other taxa while their addition allowed a more confident estimation of the southern African *Zosterops* phylogeny, a gene tree phylogeny that is congruent with the estimated species tree.

A combined 2672 bp data set for 45 individuals (including two Asian outgroup species: *Z. erythropleura* and *Z. splendidus*) was also used to corroborate the phylogenetic relationships, and was analyzed using Bayesian and Likelihood methods. Although it has been shown that increasing sample size and the number of loci used in phylogeny reconstruction (Maddison and Knowles 2006) can give a good approximation of species history when all loci are combined, species tree reconstruction has proved to be favourable and more accurate at estimating divergence times (Knowles 2009b, Heled and Drummond 2010, McCormack *et al*. 2011). The species tree topology was supported by that of the combined gene tree, although support for a split between *Z. capensis* and *Z. virens* was low. Divergence time estimates, estimated *Z. pallidus* to have shared a common ancestor with the other three taxa ~ 770 000 ya, whereas a more recent split (~ 300 000 ya) is predicted to have occurred between *Z. senegalensis* and *Z. capensis*/*Z. virens*.

The phylogenetic relationships and timing of diversification among these *Zosterops* taxa estimated here provides initial hypotheses regarding the dispersal route/s of these taxa from East Africa into southern Africa. Evidence of hybridization and introgression between *Z. pallidus* and *Z. capensis* has also been revealed, and is likely to be due to secondary contact between these taxa. Environmental analyses of sampling localities revealed that at localities of overlap, climatic conditions were intermediate between those of pure *Z. pallidus* and pure *Z. capensis* localities.

The taxonomic relationship estimated by the molecular loci supports the *Zosterops* relationships inferred from the vocal data. The Orange River White-eye *Z. pallidus* is sister to the other *Zosterops* taxa, with *Z. senegalensis* sister to *Z. capensis* and *Z. virens*. According to the molecular data, both *Z. pallidus* and *Z. senegalensis* can be classed as discrete entities, while divergence is likely to have been recent between *Z. capensis* and *Z. virens*.

6.4. Molecular phylogeography

The molecular phylogeny provided further evidence regarding southern African *Zosterops* taxonomy as well as preliminary evidence of the evolutionary history of each taxon. The application of population genetic, coalescent analyses and environmental data, in conjunction with increased sampling allowed a more in depth exploration of potential factors driving current phenotypic diversity.

Two aspects of southern African *Zosterops* evolutionary biology are explored. Firstly, whether the association of each *Zosterops* plumage type to particular Biomes within the region plays a major role in shaping the phenotypic diversity observed. Secondly, with the use of both mtDNA and nuDNA data, the extent of hybridization and introgression between *Z. pallidus* and *Z. capensis* discovered in the molecular phylogeny are established, as well whether similar patterns are evident among other taxa.

Biomes failed to explain the observed *Zosterops* phenotypic diversity found in the region, however; climatic conditions were able to group phenotypic taxon localities with localities of overlap showing intermediate characteristics of parental collecting localities. As the neutral genetic data were unable to pick up an association between phenotype and locality environmental conditions, it suggests that the impacts of climatic conditions affecting *Zosterops* structure may be a recent phenomenon. Until further work exploring selection on genes driving plumage polymorphisms in *Zosterops* is done, historical divergence of these *Zosterops* taxa is concluded to been the main mechanism for explaining the observed phenotypic diversity.

As expected, even with an increased sample size, parsimony networks for each locus mimicked those presented in the molecular phylogeny, but showed interesting placement of individuals sampled in areas of contact. Across all loci (barring CHD1), most individuals collected in contact zones tended to group with pure *Z. pallidus* individuals. A combined nuDNA Bayesian structure analysis provided further evidence of introgression of *Z. pallidus* nuDNA into *Z. capensis* and *Z. virens* individuals. This result was corroborated with an IMA coalescent analysis between *Z. pallidus* and *Z. virens* indicating asymmetrical gene flow from *Z. pallidus* into *Z. virens*. However, while IMA results were not obtained between *Z. capensis* and *Z. pallidus*, coalescent MDIV analyses revealed the presence of gene flow among all molecular loci between these taxa. Future IMA analyses will be conducted, but it is hypothesized that similar directions of gene flow found between *Z. pallidus* and *Z. virens* will be found between *Z. capensis* and *Z. pallidus*. Little evidence of gene flow was found between *Z. virens* and *Z. senegalensis*, providing support for reproductive isolation between these two taxa.

Except for a putative grouping of Western Cape *Z. capensis* individuals, no other genetic structure was observed within any of the *Zosterops* taxa, suggesting a lack of

phylogeographic breaks within each taxon's distribution range. Population expansion was shown to have occurred at some of the molecular markers in the *Z. capensis* and *Z. virens* populations. Cycles of alternating warm, wet and cool, dry periods over the past 600 000 years would have resulted in the expansion and contraction of Fynbos, Forest and Savanna Biomes. The associated population growth of *Z. capensis* and *Z. virens* during periods of habitat expansion may have resulted in recent secondary contact with *Z. pallidus*; however, large confidence intervals of divergence time estimates from coalescent analyses mean these conclusions are speculative at present.

6.5. Hypotheses revisited

Hypotheses investigated

1) *Is there any congruent morphological and genetic spatial pattern of structure within southern African* Zosterops *spp.?*

No morphological spatial structure was observed within any of the southern African *Zosterops* species. Morphometric and plumage analyses revealed four distinct morphospecies, which correlated predominantly with underpart plumage colouration. There was also no major genetic spatial structure within southern African *Zosterops* taxa. Only *Z. capensis* showed any semblance of within species structure, with populations from the Western Cape grouping together, although support for this was low.

2) *If there is spatial genetic structure, do phylogeographic breaks correspond to changes in environmental features?*

The putative phylogeographic break separating *Z. capensis* individuals falls roughly at the boundary between winter and summer rainfall, and where the Fynbos Biome changes into the Thicket and Savanna Biomes. There is no phenotypic change at this boundary and this may

therefore be a case of a cryptic taxon. Low support for this genetic break indicates that divergence within *Z. capensis* is fairly recent; however, the addition of fine scale genetic markers (i.e. microsatellites) may add additional support to the results presented here.

3) *Have past climate changes (Plio-Pleistocene) shaped genetic variability in southern African* Zosterops*?*

The divergence of *Z. pallidus* from the other *Zosterops* taxa took place ~ 770 000 ya, during the mid-Pleistocene, as did the divergence of *Z. senegalensis* from *Z. capensis* and *Z. virens* (~ 300 000 ya). Open, arid conditions are proposed for Africa near 1 000 000 ya (deMenocal 1995, 2004), followed by glacial and interglacial fluctuations every 100 000 years with these alternating cycles hypothesised to be important for faunal diversification and speciation (Variability Selection Hypothesis *sensu* deMenocal 2004). The timing of these changes in climate and environment correspond broadly with the divergence of *Z. pallidus*, an arid-adapted bird, as well as among the other *Zosterops* taxa.

Less is known about the mechanisms that may have promoted the diversification of *Z. senegalensis* from *Z. capensis* and *Z. virens*. At present, *Z. senegalensis* and *Z. virens* are ecologically separated (Moreau 1957) and this may provide insight into the factors that have promoted the initial divergence of these taxa. During a period of cool and dry conditions where open savanna predominated, the ancestor of *Z. capensis* and *Z. virens* may have found refuge in forest patches or along coastal forest which was more widespread before dispersal south along the southeastern African coast (Lawes *et al*. 2007). At the same time, *Z. senegalensis* may have remained in the drier savanna environment, one of the habitats it currently occupies along with forest further north. Further sampling of *Z. senegalensis* will provide better insight into the phylogeographical history of this taxon.

4) *How have southern African* Zosterops *spp. responded to environmental change during the Holocene?*

The forest and savanna distributed *Z. virens* shows evidence of population expansion at three of the five loci used in this study. Coalescent analyses revealed that the effective population size of this taxon is larger than *Z. capensis* and their common ancestor, also suggestive of population expansion for *Z. virens*. Warmer and wetter climatic conditions since the last glacial maximum (~ 18 000 ya) has resulted in an increase of forest habitat (Eeley *et al* 1999, Lawes *et al* 2007) which would have facilitated the expansion of *Z. virens*.

Environmental evidence presented here shows that each of the four *Zosterops* morphotypes are found to occur in distinct climatic envelopes, possibly a recent phenomenon as this is not represented by the neutral genetic data used in this study. Thus, it appears that environmental changes that have resulted since the Holocene (~ 10 000 ya) have played a role in shaping the distribution of southern African *Zosterops* taxa. It is not known however, if these effects have driven the observed phenotypic diversity, or whether this diversity arose prior to the climatic conditions experienced by each taxon at present.

5) *Do subspecies of* Z. pallidus *and* Z. virens *form monophyletic groups?*

The subspecies of *Z. pallidus* do not form monophyletic groups. This arid-adapted taxon is restricted to the wooded vegetation found along river courses throughout its range, which has provided corridors of gene flow between populations. Multivariate morphospace analyses did not reveal any substructure within *Z. pallidus*, and while the mtDNA reveals that the taxon is monophyletic there is no evidence of geographic structuring.

Clear plumage differences split *Z. virens* into two subspecies; however, the vocal data does not show any differences between these subspecies. There is evidence that grey-bellied birds from the Western Cape are on their own evolutionary trajectory, although given that

support for this is low the addition of fine-scale molecular data (i.e. microsatellites) will need to be added to test this hypothesis further. As such, the proposed subspecies of *Z. virens* do not form true monophyletic groups.

6.6. Final taxonomic conclusions

Any body of work attempting to categorize the taxonomic and species boundaries of populations will have to enter the long standing debate of species concepts, but the authors will essentially have to make up their own mind as to which concept they wish to follow. Multiple species concepts have been put forward (see Hey 2001 and de Queiroz 2005 for a thorough list and explanation of most concepts) each with their pros and cons, with advocates of each concept often finding flaws in other concepts while often overlooking the flaws in their own concept (e.g. McKitrick and Zink 1988, Cracraft 1987, Zink and McKitrick 1995, Zink 2006, Winker 2010). In ornithology, the Biological Species Concept (BSC; Wright 1940, Mayr 1942, Dobzhansky 1950) and the Phylogenetic Species Concept (PSC; Nelson and Platnick 1981, Cracraft 1983, Nixon and Wheeler 1990) are the two most widely used species concepts (Winker *et al*. 2007, Winker 2010). The BSC, based on reproductive isolation, has been criticized for not being applicable to asexual organisms, for its difficulty in applying it to allopatric populations and for not taking into account hybridization. The PSC, which recognises historically related groups as species, has also been criticized by not having a level of diagnosability that is needed to delineate species and by the fact that even individuals can be found to be diagnosable; this is a misinterpretation, however, as diagnosability criteria are intended to be applied to populations and not individuals.

With multiple processes driving the evolution of a species, and the biological complexity involved, the simplified nature of species concepts will fall short of being able to fully explain/describe species boundaries (Avise and Wollenberg 1997). Recent attempts to

bring an end to this ongoing debate have reverted to what all biologists attempt to define when describing species, and that is to identify separately evolving lineages or populations (Hey *et al.* 2003, de Queiroz 2007). Instead of attempting to define species according to the criteria proposed by any of the species concepts available, these criteria (i.e. monophyly, diagnosability, reproductive isolation, behaviour etc) should be used as "secondary defining properties" (de Queiroz 2007) to determine the level of species divergence/separation. The use of multiple criteria or characters is not a new method of species discovery, and has been suggested for many years (e.g. Hillis 1987, Crowe 1999) and should be the methodology used in future systematic research for a more holistic approach to species discovery (i.e. Tobias *et al*. 2010a).

This study has drawn together phenotypic (morphometric and plumage colouration), behavioural (songs and contact calls) and genotypic (mitochondrial and nuclear DNA) data sets to establish the taxonomic relationship of southern African *Zosterops*. Although not explicitly incorporated into species delimitation, ecological components (i.e. habitat/biome affects on geographic distribution) have been taken into account and degrees of interbreeding between potential species have been inferred from coalescent analyses.

6.6.1. Southern African Zosterops *taxonomy*

Past classifications have named and described multiple southern African *Zosterops* species and subspecies (Chapter 1), a trend commonly experienced in other *Zosterops* taxa distributed in other geographic regions (Mees 1953, Warren *et al*. 2006, Moyle *et al.* 2009, Clegg and Phillimore 2008, Milá *et al.* 2010). The use of multiple lines of evidence in this study has provided clearer insight and a more complete understanding of the white-eye taxa of the region.

The first taxon to be recognized as full species is the Orange River White-eye *Zosterops pallidus*. This group of birds is distinct from all other *Zosterops* taxa in the region according to all evidence (barring morphometric measurements) used in this work. Although evidence of hybridization with the Green Cape White-eye *Z. virens virens* (see below) exists, *Z. pallidus* individuals possess cinnamon flanks and white bellies (Chapter 2), making them phenotypically distinct from the other taxa. From a behavioural perspective, song and contact call data are significantly different (Chapter 3) from all other taxa. The vocalization data shows high congruence with the molecular phylogeny constructed using mitochondrial and nuclear markers (Chapter 4). The sister placement of *Z. pallidus* to the other southern African *Zosterops* taxa contradicts previous classifications which either lumped it with (Clancey 1967), or regarded it as sister (Moreau 1957, Skead 1967) to both forms of the Cape White-eye. The vocalization (contact calls) and molecular data (Chapter 5) also indicate hybridization of *Z. pallidus* with both *Z. capensis* and *Z. virens* in the southeastern and northeastern Free State respectively. Further investigation into hybrid zones identified during the course of this study will aim to establish whether reproductive barriers are failing in areas of contact, and if so, whether environmental or biological (or a combination of both) variables are responsible for interbreeding between these non-sister species. None of the lines of evidence examined here point to any geographic structuring within *Z. pallidus*. Thus the proposed subspecies *Z. p. deserticola*, *Z. p. sundevalli* and *Z. p. haigamchabensis* (Bowie 2005) can be rejected, with the recognition of the Orange River White-eye as a monotypic species: *Zosterops pallidus* Swainson (1838).

Firm conclusions on the species status of the African Yellow White-eye *Z. senegalensis* cannot be made from the evidence presented in this study as molecular data are lacking from much of its distribution. Morphometric analyses (Chapter 2) revealed that *Z. senegalensis* birds had longer bills, but shorter wings and tails in comparison to the other

Zosterops taxa, whereas plumage colour measurements indicate a split within *Z. senegalensis* into green- and yellow-bellied individuals which likely correspond to subspecies. Song data (Chapter 3) do, however, not discriminate between *Z. senegalensis* and both forms of the Cape White-eye. The molecular phylogeny (Chapter 4), although only including samples from Malawi, shows that the African Yellow White-eye is sister to both forms of the Cape White-eye and that divergence between them took place fairly recently. This recent divergence is likely to account for the lack of divergence in song characters observed and also for the presence of gene flow (albeit very low levels and with a low sample size of *Z. senegalensis* individuals) with the Green Cape White-eye *Z. v. virens* in the coalescent analyses (Chapter 5). No records of interbreeding and hybridization have been reported between *Z. senegalensis* and any of the other southern African *Zosterops* taxa, with differences in habitat (thornveld vs forest; Moreau 1957) suspected to be the reason for preventing taxa from coming into contact. Multiple subspecies of the African Yellow White-eye *Z. senegalensis* are currently in use (Smith 2005, van Balen 2008), however, insufficient sampling of molecular data across the species' range does not allow for any conclusions regarding the subspecies nomenclature of the species. Vocalization data also did not reveal any evidence of subspecific differences within *Z. senegalenesis*, not surprising as no differences could be found between *Z. capensis*, *Z. senegalensis* and *Z. virens*. The morphological data sets, and in particular underpart plumage colouration, do indicate the presence of green- and yellow-bellied subspecies within *Z. senegalensis*. Further sampling to obtain additional morphological, vocalization and molecular character information is needed before a complete picture of *Z. senegalensis* taxonomy can be reached. As such, the continued recognition of *Z. senegalensis* as a separate species should be accepted until this further information can be obtained and incorporated into what is presently available.

A clear morphological distinction (Chapter 2) exists between the Cape White-eye *Z. capensis* and the Green White-eye *Z. virens*, underpart plumage colouration being grey and green respectively with no clinal variation. Song and contact call data (Chapter 3) reveal no differences, thus no taxonomic distinction between the taxa can be made using vocalization data. The observed paraphyly of *Z. capensis* individuals in the combined molecular phylogeny (Chapter 4) is likely due to incomplete lineage sorting and suggests recent divergence of *Z. capensis* and *Z. virens*. Although tentative, coalescent analyses (Chapter 5) suggest a divergence date of ~56 000 ya, but more interesting, that high levels of gene flow still exist between these taxa, at points of contact between taxa where individuals of intermediate phenotypes occur.

Changes in underpart plumage colouration have still occurred despite the high levels of gene flow observed, a case of phenotypic divergence with the presence of gene flow as opposed to the homogenizing affect that high levels of gene flow are expected to have on populations. Such phenomena are not new though, with plumage polymorphism in the presence of gene flow shown to occur in other avian taxa (Cooke *et al.* 1988, Antoniazza *et al.* 2010, Lim *et al.* 2010, Pryke 2010, Ribeiro *et al*. 2011), as well as in *Zosterops* (Milá *et al.* 2010) taxa. Local adaptation to the contrasting habitats where *Z. capensis* (Fynbos) and *Z. virens* (coastal Forest and Savanna) occur is likely to account for the rapid change in plumage polymorphism observed. Further work to identify the gene/s responsible for underpart plumage colouration will provide additional information regarding the variation observed in southern African *Zosterops* and potentially for other *Zosterops* taxa.

Although large morphological divergence is observed between *Z. capensis* and *Z. virens*, similarities in vocalizations together with genotypic cohesion suggests a recent divergence of these taxa. Thus, with the evidence presented from this study, these two

Zosterops taxa are considered subspecies of the Cape White-eye: *Zosterops virens capensis* Sundevall (1850) and *Zosterops virens virens* Sundevall (1850).

6.7. Future prospects

The taxonomy and evolutionary relationships of southern African *Zosterops* completed in this dissertation has exposed other avenues of scientific enquiry, especially focused on mechanisms of speciation, which can be explored using this system.

Areas of contact between the Orange River White-eye *Z. pallidus* and both sub-species of the Cape White-eye *Z. virens* have been identified during the course of this study. In particular, *Z. pallidus* and *Z. v. capensis* come into contact and hybridize in the southeastern Free State, whereas *Z. pallidus* and *Z. v. virens* hybridize in the northeastern Free State. Hybrid zones are beneficial to evolutionary biologists in that they provide an opportunity to determine various mechanisms driving the diversification of taxa (Barton and Hewitt 1985, Hewitt 1988). Comparisons of conditions found in zones of contact with those found in pure parental ranges (i.e. core *Z. pallidus* and *Z. virens* ranges) may provide evidence of factors that may have caused *Zosterops* divergence, and that may breakdown in areas of contact facilitating interbreeding between separate species. Intermediate vegetation structure, climatic conditions, or physiological responses in hybrid zones will provide the first indicators as to the factors behind interbreeding in these areas. Additionally, playback experiments using song and call recordings will determine if there is a breakdown in behavioural and reproductive isolating mechanisms in areas where separate species come into contact.

Various methods and approaches can be employed to uncover the genetic basis of adaptive traits that are important in determining how populations adaptively diverge and eventually give rise to new species, where different environments may cause ecological

selection which may in turn lead to local adaptation. Whole genome scans using AFLP (Wood *et al*. 2008, Nunes *et al.* 2011) or microsatellite (Schalkwyk 1999) or SNP (Slate *et al*. 2011) markers can be used in non-model organisms to search for outlier loci (Luikart *et al*. 2003, Nosil *et al*. 2009). Outlier loci deviate from neutral marker expectations due to influences of selection, and can be linked to environmental and other selection pressure to determine which loci are important for divergence between taxa (Joost *et al*. 2008, Nunes *et al*. 2011). Another emerging field that attempts to understand causes of speciation is that of looking for speciation genes (Butlin and Ritchie 2001, Orr *et al*. 2004, Wu and Ting 2004). Most speciation gene research has naturally been done on model organisms such as *Drosophila* species (Presgraves *et al*. 2003, Barbash *et al*. 2003, Wu and Ting 2004) However, recent work in house mice (*Mus domesticus*) have discovered genes responsible for hybrid sterility between subspecies (Mihola *et al*. 2009). Although no similar research has been conducted for any bird taxa, the Z-chromosome is a suggested starting point to look for a bird speciation genes. In female-heterogametic species (such as birds), the Z-chromosome could be important in adaptive speciation with linkage disequilibrium between genes for female mate preference and male sexual signals likely to be maintained even though gene flow could be present (Qvarnström and Bailey 2008).

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