UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

# The taxonomic status of the Damaraland Redbilled Hornbill (*Tockus erythrorhynchus damarensis*)

## A behavioural, morphological and molecular analysis

Wayne Delport

Department of Zoology and Entomology
University of Pretoria
Pretoria
South Africa

A thesis submitted to the Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria in fulfilment of the requirements for the degree Magister Scientia

Pretoria 2001

© University of Pretoria

**Declaration**

I declare that this thesis is my own, unaided work. It is submitted as the requirement for the degree Magister Scientia at the University of Pretoria, Pretoria. It has not been submitted before for any degree or examination at any other university.

---

Signed on this 1st day of August 2001

*For my parents*
*Thanks for the unconditional love and support*

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

# Contents

# Acknowledgements

First I would like to take this opportunity to thank my supervisors Willem Ferguson, Alan Kemp and Paulette Bloomer who have provided unparalleled guidance for the duration of this MSc. At the onset of this project I took on the near-lunatic approach of applying for financial assistance from every company that would putatively support a 'conservation project on the Damaraland Redbilled Hornbill'. The success rate was low but nonetheless I would explicitly like to thank Total Fuel Namibia for fuel, Mondi Timber for the wood used to build nestboxes and Plascon Paints for supplying paint to make the nestboxes weatherproof. Furthermore the Mazda Wildlife Fund and Humphrey le Grice supplied a Mazda Double-Cab 4x4 for two of the six field trips to Namibia. I remain on the topic of fieldwork and would like to thank Joris Komen, John Mendelsohn and Rob Simmons for advice on where to find the contact zone between two Redbilled Hornbill subspecies. Also thanks to Rob for his assistance in getting me past border patrol with a truck load of nestboxes. Apparently research constitutes work and therefore both research and work permits are required. Thanks also to those border patrol officials who have taught me so much about humanity in the 20$^{th}$ century. The Namibian Ministry of Environment and Tourism were professional when they granted research permits. Thank you. I wish I could say the same of the Ministry of Home Affairs with regard to work permits. However one person did shine through as a saviour to a biology research student: thank you Mrs Diedriechs. Again on the topic of fieldwork I would like to thank all the farmers who granted me permission to work on their lands. Leonard Amabub, Hannes Richter, Chris Coetzee, Wayne McAdam and Arno Pusch. Furthermore Arno and his wife Karien provided me with a bed, a shower and good German cooking. Thanks Arno, thanks Karien. Tony Knowles helped set up the nestbox study site, thanks boet. Christiaan Boix was always a good hornbill-guru to overnight with on the way to my field site. Thank-you Christiaan for good discussions, good advice and always a good pint or two. In addition I would like to thank Christiaan for allowing me to use his Daan Viljoen Nestbox study site to obtain some hornbill blood samples. At the completion of fieldwork Joe's Beer House had the best gemsbok fillet. Back in South Africa the Transvaal Natural History Museum, Pretoria and its staff provided access

to both sound and skin collections. Thank you to the curators Laura Straton & Tamar Cassidy for their assistance and especially for the loan of good binoculars and sound recording equipment. Furthermore I would like to thank the following contributors to the Fitzpatrick Sound Collection housed at the Transvaal Museum: M. Blom, J. B. Dunning, W. D. Keibel, K. Newman, O. M. Prozesky, P. Sellar, J. Stannard, S. E. van Mienop, A. van Zyl & A. Walker. With regard to field work in South Africa I would like to thank Fan and Annette Malign who allowed me to catch hornbills on their Farm in the northern Province, South Africa and to Kyriakos Tsimboukis who supplied me with a Redbilled Hornbill sample from Swartwater, Northern Province. Also thanks to C. R. Barlow who sent a tissue sample of a Gambian Redbilled Hornbill to Alan Kemp. In the laboratory I would like to thank Rentia Tukker who started training me in molecular methods and Cathi Lehn who completed the task. Thanks also to Cathi for breaking lab monotony whilst she was in South Africa. Pam de Waal helped me through eight months of unsuccessful cloning attempts and Tyron Grant helped with the pure mtDNA extractions. Thanks to both of you, I have learnt so much. Finally I have left my family for last. My parents and brother, Sean, have been supportive and understanding. Thank-you. Also thanks Dad for the vehicle in my last field season. Also thanks to Sean and my girlfriend, Linda, for all those weekends of building nestboxes. As much as they might have complained I know they enjoyed it. Linda, you have been extremely supportive, even in light of those long fieldtrips away. Thank-you my love. In conclusion I would like to thank the Lord for blessing me with a thirst for knowledge and an appreciation of nature.

# Chapter 1

## Introduction

No one definition has as yet satisfied all naturalists; yet every naturalist knows vaguely what he means when he speaks of a species

Darwin 1859

The natural world is complex and comprises great biological diversity. As a consequence biologists, in their inherent desire to classify, have subdivided this biodiversity into kingdoms, phyla, orders, families, genera, species and subspecies. However, some have questioned the validity of each of these taxonomic ranks (Sylvester-Bradley 1956; Bonde 1981 in Nelson 1989) and some have concluded that the only real biological unit is the species (Raven 1974; Løvtrup 1987 in Nelson 1989). As a result the debate over species concepts has proliferated in the literature for several decades. The original proponents of the Biological Species Concept (Dobzhansky 1940, Mayr 1970) defined species as groups of populations that are reproductively isolated from one another. This isolation is proposed to be the result of isolation mechanisms that have evolved for the purpose of preventing mating between different species (Dobzhansky 1970). The obvious philosophical problem with the BSC is that, if speciation occurs via allopatry how do these isolating mechanisms evolve for the purpose to prevent mating between geographically subdivided populations. This philosophical problem of the BSC made way for a new species concept. Paterson's (1985) Recognition Species Concept defines species as populations that share a common fertilisation system. However, such a system has arisen for the purpose of communication within species and has prevention of mating between species as a fortuitous consequence. The proliferation of phylogenetics, and the need to produce species taxa compatible with such analyses, led to the development of another species concept by Cracraft (1989); termed the Phylogenetic Species Concept. The PSC defines species as populations of organisms that have consistently diagnosable characters, whether morphological or molecular. Templeton (1998) and Ferguson (1998) have both considered the differences between the above species concepts. It is clear that both the Biological and Recognition Species Concepts deal with processes, i.e. they are species concepts that define species from the process of speciation. However Cracraft's (1989) PSC deals with the culmination of such processes, the patterns of evolutionary lineages. It is this combination of pattern and process that has resulted in Templeton's (1989) Cohesion Species Concept. Templeton's (1989) Cohesion Species Concept comprises a series of testable hypothesis that deal with diagnosability on morphological and molecular grounds, gene flow between populations and hybrid infertility. Finally, Crowe (1998) has discussed a multifaceted approach to determining species status, where

species that are phylogenetically compatible are defined through a collaboration of several data sources. I do not want to take this opportunity to claim support for any of the species concepts outlined but rather to discuss the philosophy behind this research on the African Redbilled Hornbills (*Tockus erythrorhynchus* subspecies complex).

The African Redbilled Hornbill subspecies complex (*T. erythrorhynchus*) comprises six subspecies (Figure 1) that are distinguishable on the basis of morphological features (Kemp 1995, Delport 1999). These features are eye colour, facial plumage colour and circumorbital skin colour. This research project set out to evaluate whether two of the subspecies, *Tockus erythrorhynchus rufirostris* and *Tockus erythrorhynchus damarensis*, could be considered independent species. Obviously the species concept that one adopts at onset of such research is likely to affect the type of data collected. If I adopted a Biological Species Concept, communication and breeding pair composition data from a contact zone would have been sufficient to confirm or reject the hypothesis at hand. Furthermore, if I adopted the approach of the Recognition Species Concept I would have collected data on the putative specific-mate-recognition-systems of the two subspecies and made decisions on that basis. Finally, if I adopted the approach of the Phylogenetic Species Concept the analysis of molecular and morpho-behavioural variation between allopatric populations of the two subspecies would have been sufficient to reject or accept the hypothesis. Instead of adopting the approaches of any of these concepts, I set out to collect data that my supervisors and I intuitively felt were important to determine taxonomic status. The research has thus produced the following chapters of this thesis.

**Chapter 2: Geographic call variation between subspecies of the African Redbilled Hornbills (*Tockus erythrorhynchus* subspecies)**

This chapter explores geographic variation in the vocalisations of four of the subspecies of Redbilled Hornbill. The work is essentially taking the communication components of the Biological and Recognition Species Concepts and determining whether there may be a mechanism or communication system in place that may prevent mating between the subspecies of Redbilled Hornbill. Furthermore, chapter 2 introduces the idea that there may be hybridisation between two of the subspecies in northern Namibia.

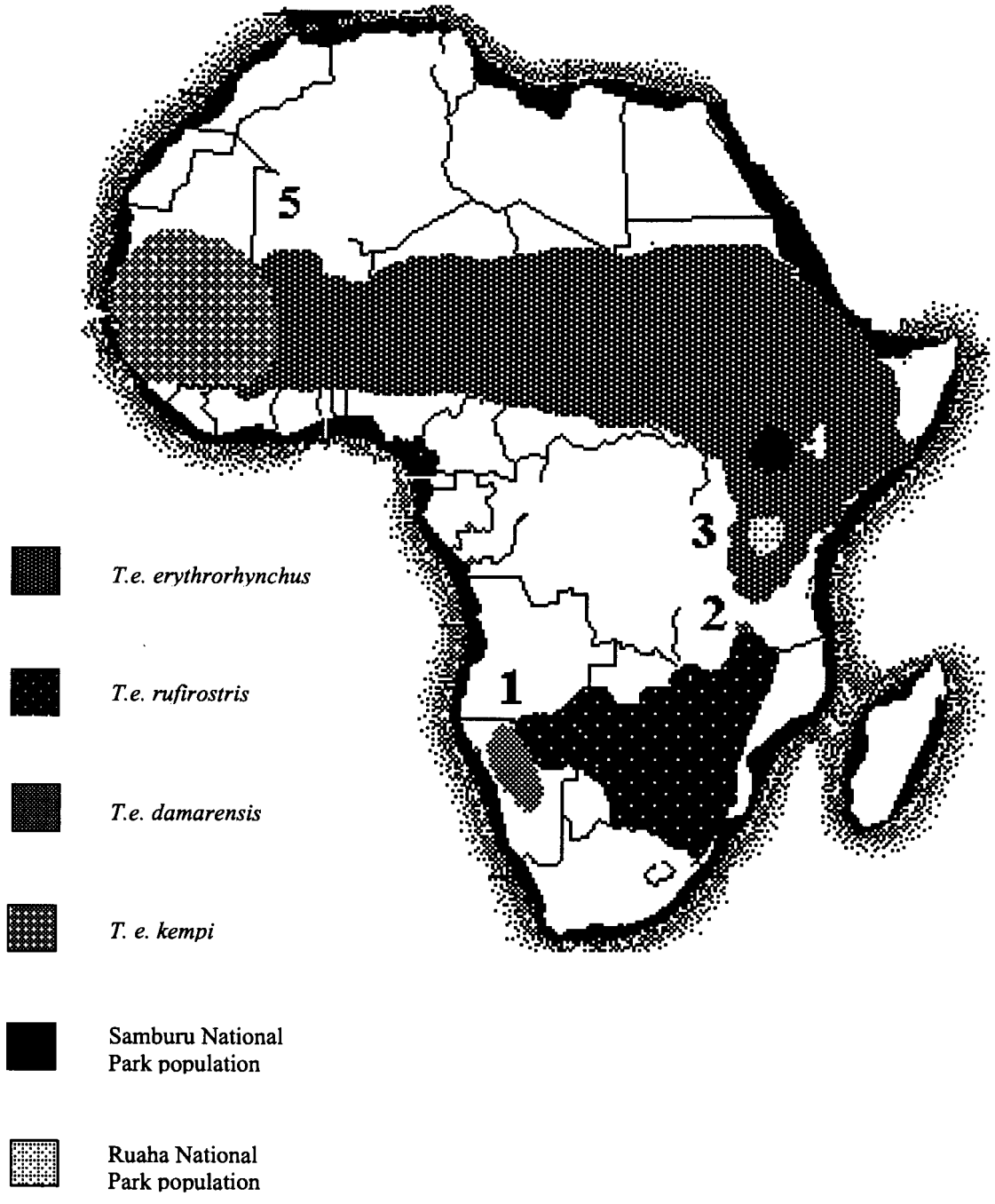



**Figure 1:** Approximate distributions of the subspecies of Redbilled Hornbills (Tockus erythrorhynchus). Numbers 1-5 indicate confirmed (1) or possible (2-5) zones of hybridisation.

**Chapter 3: The structure of an African Redbilled Hornbill hybrid zone revealed by morphological, behavioural and breeding biology data**

Chapter 3 takes the observation of hybridisation in the northern Namibia contact zone further. In this chapter, I analyse call structure and morphological differences geographically across the contact zone and demonstrate that it resembles a typical cline between two species. In addition, I quantify the breeding success of homo- and heterospecific pairs and evaluate whether the observed breeding pair composition is the result of random mating.

**Chapter 4: Characterisation and evolution of the mitochondrial DNA control region in hornbills (Bucerotiformes)**

Chapter 4 is a spin-off of the molecular work and explores the evolution of the mitochondrial DNA control region in the Bucerotiformes. The hornbill control region is longer than that of other birds and contains a series of 23bp repeats. These repeats have been identified in the chicken (*Gallus gallus*) but the nature of the repeat differs in hornbills. Therefore, I explore the putative origin of these repeats in hornbills and discuss their relationship to the control region repeats found in other birds.

**Chapter 5: Mitochondrial DNA gene flow between two morphologically distinct subspecies of African Redbilled Hornbill (*Tockus erythrorhynchus*) and implications for taxonomic status**

Finally, chapter 5 discusses the molecular variation both within and between the two subspecies of Redbilled Hornbill. If I adopted a Phylogenetic Species Concept this may have been sufficient to identify taxonomic status. However, the presence or absence of gene flow in a contact zone would be necessary to include when deciding taxonomic status. Therefore, this chapter explores gene flow between allopatric and sympatric populations of the two subspecies of Redbilled Hornbill considered.

Please take note that each chapter has been written as an independent manuscript and there may be repetition between chapters. This repetition is intended to enhance the

independent readability of each manuscript. Furthermore, I refer to 'we' instead of 'I' in all of the chapters since each chapter will be submitted for publication with my supervisors as co-authors. Moreover, I use the terms homo- and heterospecific loosely throughout the thesis. In this case it would probably be more appropriate to use homo- and heterosubspecific, but these are very wordy and reduce readability.

The completion of this MSc has enabled one to decide on the taxonomic status of two subspecies of Redbilled Hornbill, *Tockus erythrorhynchus rufirostris* and *Tockus erythrorhynchus damarensis*. However four subspecies remain and their phylogenetic relationships still need to be determined. Therefore, the end of this MSc research project is not necessarily an end in itself but rather a commencement on an intriguing field of biological research.

# Chapter 2

## Geographic call variation between subspecies of the African Redbilled Hornbill (*Tockus erythrorhynchus* subspecies)

"Geographic speciation is thinkable only, if subspecies are incipient species. This of course, does not mean that every subspecies will eventually develop into a good species. Far from it! All this statement implies is that every species that developed through geographic speciation had to pass through the subspecies stage."

Mayr 1942

**Abstract**

Geographic variation of bird vocalisation has been studied extensively in the passerine birds. Non-passerines on the other hand are generally thought to exhibit limited geographic variation due to the supposed genetic inheritance of their calls. We consider geographic call variation in the non-passerine African Redbilled Hornbills (*Tockus erythrorhynchus* subspecies) through a quantitative analysis of 12 call variables. A forward Discriminant Function Analysis of four morphologically and behaviourally distinct subspecies of Redbilled Hornbill (*T. e. rufirostris*, *T. e. damarensis*, *T. e. erythrorhynchus* and *T. e. kempi*) as well as *Tockus monteiri*, a close relative, yielded a total classification success of 82.61%. All the pairwise classifications exceeded 95% success except that between *T. e. rufirostris* and *T. e. damarensis* (79.45%). The misclassifications probably result from hybridisation in a contact zone between *T. e. rufirostris* and *T. e. damarensis*. The vocalisation differences between *T. e. rufirostris* and *T. e. damarensis* are constant between allopatric populations that are representative of the geographic ranges of these two subspecies. Therefore, constant differences between the calls of the African Redbilled Hornbills could warrant each of the six subspecies specific status. However, an analysis of gene flow between the divergent populations of Redbilled Hornbill would lend support to our argument for specific status.

## Introduction

Geographic variation of bird vocalisation has been considered by several authors (see Thielcke 1969, Mundinger 1982 for reviews). However, most authors have worked with passerines, particularly oscines (Mundinger 1982, Kroodsma *et al.* 1984, Bretagnolle & Genevois 1997) which have the ability to learn vocalisations and thus produce songs that are strongly influenced by the environment (Kroodsma and Baylis 1982). As a result, many oscine species, having several pairs of syringeal muscles, exhibit a greater capacity for vocal differentiation (Lanyon 1969) and for geographic variation in their vocalisations (Marler & Tamura 1962, Byers 1996, Diamond 1998, Kroodsma *et al.* 1999, Tracy & Baker 1999). However, Lindell (1998) has argued that, although the absence of learning in sub-oscines removes a major source of variation, it does not prevent geographic variation from arising through restricted gene flow between populations, genetic drift or natural selection. The analysis of geographic variation in non-passerine vocalisations could therefore be used to address taxonomic and phylogenetic problems.

The African Redbilled Hornbill complex (*Tockus erythrorhynchus*) comprises four recognised subspecies and two additional undescribed variants (Kemp 1995). The North African Redbilled Hornbill *T. e. erythrorhynchus*, the southern African Redbilled Hornbill *T. e. rufirostris*, the Damaraland Redbilled Hornbill *T. e. damarensis* in Namibia and the Senegal-Gambia Redbilled Hornbill (*T. e. kempi* - Tréca & Erard 2000) exhibit morphological differences (Figure 1) that allow reliable discrimination between them. The additional populations that occur in northern Kenya and southern Tanzania also show sufficient morphological differences for reliable discrimination (Kemp 1995). In addition, Kemp (1995) suggested that there are differences in the courtship display of the races of Redbilled Hornbill. The display call includes a long series of clucking notes that accelerate and break into double-clucks (Figure 2). These double-clucks are accompanied by bobbing of the body with the head held down. The posture of the wings during this display differs among the subspecies (Figure 1). The southern African Redbilled Hornbills (*T. e. rufirostris*) display with their wings closed, the North African (*T. e. erythrorhynchus*) and Damaraland (*T. e. damarensis*) Redbilled Hornbills with their wings partially open and the Senegal-Gambian Redbilled Hornbills (*T. e. kempi*) with their wings fully

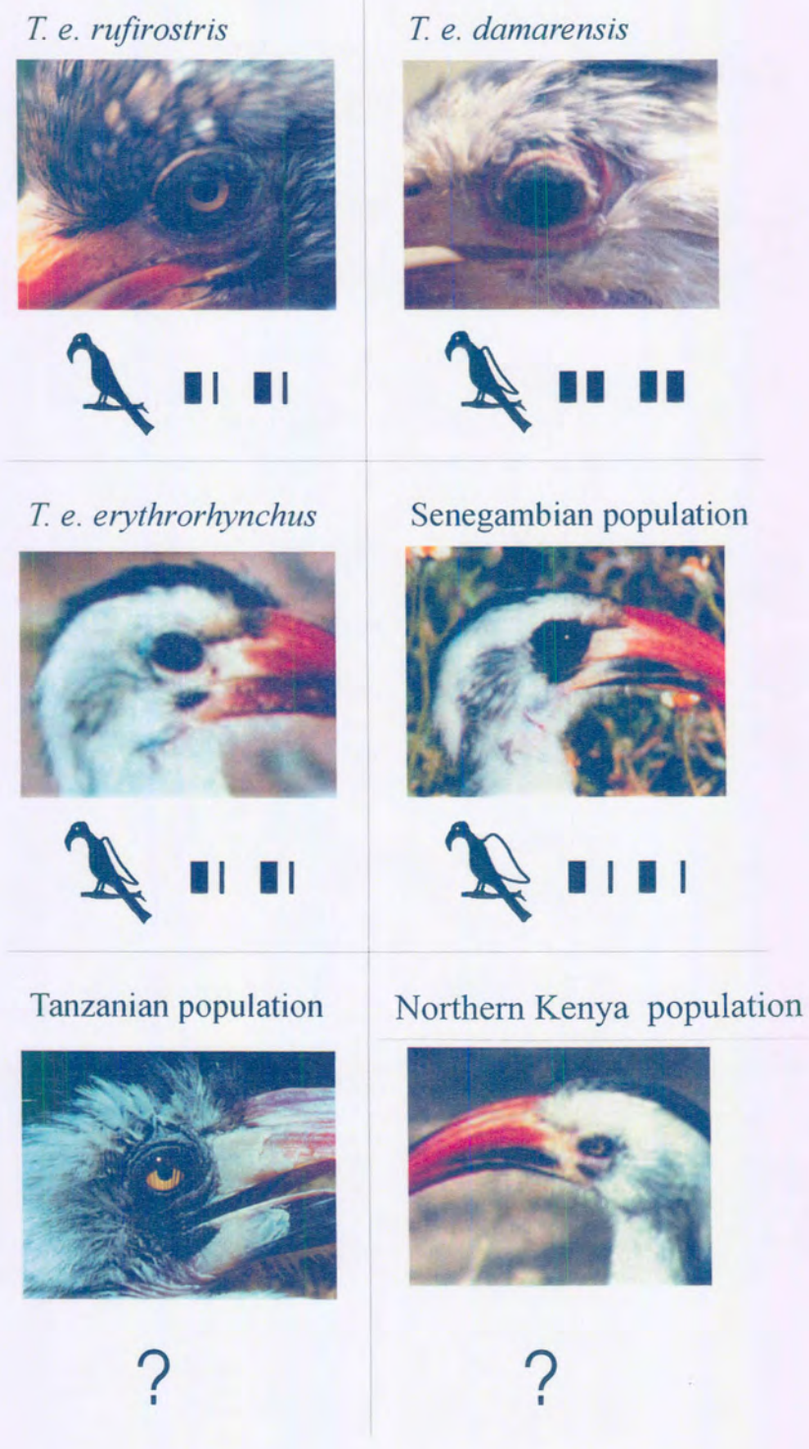



**Figure 1**: Morphological, courtship display and courtship call characteristics of the subspecies of African Redbilled Hornbill. The subspecies exhibit (i) morphological differences in eye colour and circumorbital skin colour, (ii) courtship display differences in the position of the wings, and (iii) courtship call differences in the characteristics of double-cluck notes. The courtship display and call characteristics of the Tanzanian and Northern Kenyan populations have not yet been described. Courtship display characteristics are from Kemp (1995) and call characteristics are from a preliminary visual analysis of sonograms.

open (Kemp 1995). The displays of the northern Kenyan and southern Tanzanian Redbilled Hornbill populations have not yet been characterised. Kemp (1976) showed that the differences in the courtship display of five species groups of *Tockus* hornbills (*T. bradfieldi, T. nasutus, T. hemprichii, T. monteiri and T. flavirostris*) comprise tail, head, body and wing movements. The *T. erythrorhynchus* complex falls within the *T. monteiri* species group, above. Since hornbill courtship display appears to serve as a reliable discrimination cue between the above species groups, it may also allow reliable discrimination between the taxa of the Redbilled Hornbill complex. Paterson (1985) defined a species as a group of populations of organisms that share a common fertilisation system, including a specific-mate recognition system. Indeed, species-specific signaling has also been included in species concepts such as the Biological Species Concept (Mayr 1970) and the Cohesion Concept (Templeton 1989). A courtship display that forms part of the specific-mate recognition system is therefore an excellent starting point in deciding whether divergent populations could be considered independent species. In this paper we document the geographic call variation between subspecies of the African Redbilled Hornbill. We include in the analysis the hornbill most closely related to Redbilled Hornbills, Monteiro's Hornbill (*Tockus monteiri*), to compare the differences between vocalisations of subspecies to that within a recognised species.

## Methods and Materials

*Sound acquisition*

Two subspecies of Redbilled Hornbill, *T. e. damarensis* and *T. e. rufirostris,* occur in sympatry over a narrow contact zone in northern Namibia (19° 20' S, 17° 21' E). We used a Sony TCM-38V cassette recorder with a Tect 913 directional microphone to record the courtship calls of fifteen *T. e. damarensis* and ten *T. e. rufirostris* individuals in the contact zone during the breeding season from 15 January 1999 to 1 April 1999. In addition, we recorded the calls of 34 *T. e. damarensis* individuals in allopatry at the following four localities: Outjo (20° 10' S, 16° 10' E, n = 9), Kamanjab (19° 35' S, 14° 40' E, n =3), Omaruru (21° 25' S, 15° 55' E, n = 5) and Daan Viljoen Nature Reserve (22° 32' S, 16° 58' E, n = 17), using the same equipment. Furthermore, we obtained the calls of eight allopatric *T. e. rufirostris* individuals from the FitzPatrick Sound Communication Library (FSCL), Transvaal

Museum, Pretoria, South Africa. The locality data of these eight *T. e. rufirostris* calls were not complete and therefore we could only assign the calls to the following broad localities: Zimbabwe (n = 3), Northern Province, South Africa (n = 3) and Kruger National Park, South Africa (n = 2). In addition to these calls obtained from the FSCL, we recorded the calls of eight *T. e. rufirostris* from the northeastern extreme of the Namibian contact zone. We obtained the calls of six *T. e. erythrorhynchus* individuals and eight *T. e. kempi* individuals from the FSCL. Finally four courtship calls of Monteiro's Hornbill (*T. monteiri*) were recorded at Daan Viljoen Game Reserve using the above equipment.

*Sonographic analysis*

The courtship call of Redbilled Hornbills (Figure 2) consists of a long series of single clucking notes that accelerate and break into double-clucking notes. We refer to the first part of the call as the first call component and the part after the double-clucking notes begin as the second escalated component. We digitised the recorded calls as 16 bit sound files (8 kHz sampling frequency) on an Apple Macintosh Power PC using Canary version 2.1 (Cornell Laboratory of Ornithology). We generated independent sonograms (Figure 2) for temporal and frequency measurements. Sonograms used for temporal measures had a time resolution of 2.0 ms (FFT = 256) whilst sonograms used for frequency measures had a frequency resolution of 20 Hz (FFT = 512). Twelve variables were measured from the sonograms (Figure 2, Table 1).

*Statistical analysis*

The statistical analysis we conducted aimed (i) to determine if the calls of a single subspecies were consistent between geographically isolated populations and (ii) to determine if there were significant differences between the calls of the four subspecies of Redbilled Hornbill. In the first analysis we used the 34 allopatric *T. e. damarensis* calls recorded at four localities and the 16 allopatric *T. e. rufirostris* calls also recorded at four localities. We performed a MANOVA of the 12 call variables (i) on both the *T. e. damarensis* and *T. e. rufirostris* populations combined, (ii) on only the *T. e. damarensis* populations and (iii) on only the *T. e. rufirostris* populations. In the second analysis, we used the 12 variables measured from 93 calls in a principal component analysis and a forward-stepwise Discriminant Function Analysis (DFA). These multivariate techniques were used to determine whether we could reliably

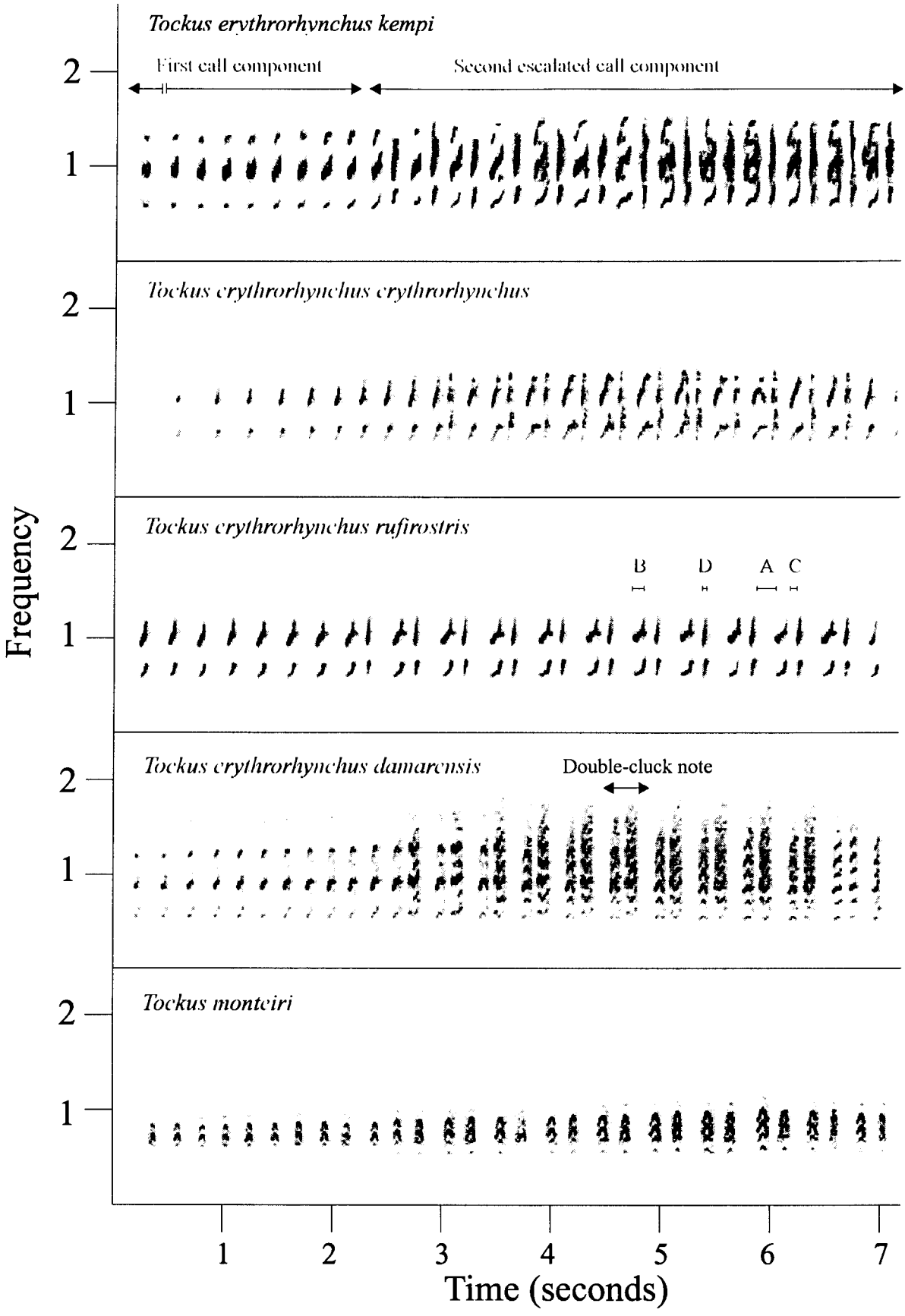



**Figure 2**: Spectrograms of the calls of four subspecies of the African Redbilled Hornbills and Monteiro's Hornbill. Some of the variables measured in the analyses are represented. The measurements A, B, C and D are repeated for all clucking sounds in the second escalated component of the call and averaged to a mean value for each individual.

**Table 1** : Variables measured from sonograms and used in discriminant function analysis of recordings from five hornbill taxa. Mean values and standard deviations (in parenthesis) are represented (*T. m* = *Tockus monteiri*, *T. e. e.* = *Tockus erythrorhynchus erythrorhynchus*, *T. e. r* = *Tockus erythrorhynchus rufirostris*, *T. e. d* = *Tockus erythrorhynchus damarensis* and *T. e. k* = *Tockus erythrorhynchus kempi*).

| Mnemonic | Variable | Units | Taxon mean (standard deviation) | | | | |
|---|---|---|---|---|---|---|---|
| | | | *T. m* | *T. e. e* | *T. e. r* | *T. e. d* | *T. e. k* |
| DUR | Total duration of call | s | 11.7 (1.5) | 6.6 (1.3) | 9.3 (2.4) | 9.4 (2.4) | 6.4 (1.1) |
| PRO_1 | Proportion of call comprising the first component | % | 35.7 (6.3) | 37.9 (6.5) | 46.5 (14.6) | 48.3 (10.5) | 34.7 (6.9) |
| PRO_2 | Proportion of call comprising the second escalated component | % | 64.3 (6.3) | 62.1 (6.5) | 49.8 (15.0) | 51.8 (10.5) | 65.3 (6.9) |
| MDUR_C1 | Mean duration of all the notes in the first call component | s | 0.077 (0.008) | 0.063 (0.008) | 0.053 (0.012) | 0.054 (0.011) | 0.074 (0.022) |
| MDUR_BC1 | Mean duration between all successive notes in the first call component | s | 0.175 (0.021) | 0.163 (0.024) | 0.159 (0.030) | 0.162 (0.032) | 0.165 (0.033) |
| N_C1 | Number of notes in first call component | n | 16.5 (1.9) | 9.5 (1.8) | 18.0 (7.35) | 21.1 (9.3) | 8.8 (3.3) |
| N_C2 | Number of notes in the second escalated call component | n | 31.8 (8.1) | 30.0 (7.7) | 34.7 (13.4) | 31.2 (9.6) | 24 (2.7) |
| A* | Mean duration within double-clucks | s | 0.085 (0.008) | 0.046 (0.005) | 0.048 (0.010) | 0.065 (0.009) | 0.080 (0.005) |
| B* | Mean duration of the first notes of double-clucks | s | 0.116 (0.014) | 0.091 (0.006) | 0.074 (0.015) | 0.072 (0.005) | 0.106 (0.010) |
| C* | Mean duration between double-clucks | s | 0.173 (0.008) | 0.093 (0.004) | 0.114 (0.021) | 0.114 (0.020) | 0.116 (0.011) |
| D* | Mean duration of the second notes of double-clucks | s | 0.111 (0.007) | 0.054 (0.003) | 0.063 (0.010) | 0.066 (0.016) | 0.052 (0.008) |
| C – A | The duration mean A subtracted from the duration mean C | s | 0.094 (0.006) | 0.057 (0.006) | 0.064 (0.016) | 0.052 (0.020) | 0.030 (0.012) |
| B – D | The duration mean D subtracted from the duration mean B | s | 0.001 (0.009) | 0.04 (0.007) | 0.016 (0.023) | 0.004 (0.013) | 0.054 (0.011) |
| C_RATE1 | Rate at which notes are uttered in the first call component | $notes.s^{-1}$ | 0.043 (0.003) | 0.046 (0.008) | 0.043 (0.008) | 0.045 (0.007) | 0.045 (0.01) |
| C_RATE2 | Rate at which notes are uttered in the second escalated call component | $notes.s^{-1}$ | 0.041 (0.003) | 0.073 (0.003) | 0.083 (0.034) | 0.065 (0.012) | 0.068 (0.008) |
| EM_FREQ | Emphasized frequency (frequency emmited at the loudest amplitude) of the call | Hz | 990.3 (93.4) | 1241.8 (17.6) | 1096.8 (182.2) | 1037.3 (154.9) | 1146.9 (91.8) |

* Measurements that were not included in the analyses, but were used to calculate some variables.

distinguish between the subspecies of Redbilled Hornbill based on the 12 variables. The calls of Monteiro's Hornbill (*T. monteiri*) were included in the analysis to provide a yardstick by which we could compare the magnitude of differences between taxa of Redbilled Hornbill to the differences between two recognised species (*T. erythrorhynchus* and *T. monteiri*). We also excluded *T. monteiri* in another analysis to determine if the resolution of the analysis could be improved. Forward-stepwise DFA also enabled us to identify which variables were most useful to discriminate between the calls of the taxa included in the analyses. We used ANOVA to test for significant differences between the means of the most useful discriminating variables of the five taxonomic groups (*T. monteiri, T. e. damarensis, T. e. rufirostris, T. e. erythrorhynchus* and *T. e. kempi*) and proceeded with a *post hoc* pair-wise comparison of means (Duncan's multiple range test).

## Results

*Within-taxon variation*

The MANOVA that included both *T. e. damarensis* and *T. e. rufirostris* populations indicated that there were significant differences between the 12 variables of some of the populations (Wilks' Lambda = 0.0073, Rao's R = 2.81, $df_1 = 84$, $df_2 = 191$, $p < 0.001$). Although MANOVA does not enable one to identify where the significant differences lie, we were able to determine if they occurred within subspecies by partitioning the analysis to include either only *T. e. damarensis* or only *T. e. rufirostris* populations. The MANOVAs that included only populations of a single subspecies yielded no significant differences among the 12 call variables.

*Multivariate analyses*

The principal component analysis extracted five principal components that described 81.66% of the total variation. This analysis technique, however, did not allow discrimination between taxa. In the forward-stepwise DFA four canonical roots accounted for 100% of the variation between groups (Canonical root 1: 59.11%, roots 1-2: 82.77%, roots 1-3: 97.40% and roots 1-4: 100%). The forward-stepwise DFA discriminated with 100% success between all the groups included in the analysis, except the *T. e. erythrorhynchus – T. e. rufirostris* and *T. e. rufirostris – T. e. damarensis* pairs (Table 2). The *T. e. rufirostris* cluster does not overlap with *T. e.*

*erythrorhynchus* in the scatterplot of the first two canonical roots (Figure 3), however these clusters do overlap in the third and fourth canonical roots (Figure 3). A high classification success is achieved between the *T. e. erythrorhynchus* and *T. e. rufirostris* clusters (Table 2). Therefore, we consider the *T. e. erythrorhynchus* cluster to be consistently different from *T. e. rufirostris*. The classification success achieved is lower (79.45%) for the clusters of *T. e. rufirostris* and *T. e. damarensis* and the clusters overlap in the scatterplot of the first two canonical roots (Figure 3). However, this overlap is reduced significantly in the scatterplot of third and fourth canonical roots (Figure 3). The analysis that excluded *T. monteiri* did not increase the resolution between the four forms of Redbilled Hornbill.

The three most useful variables identified by the forward DFA are B-D, C_RATE2 and C-A (Table 3). The principal component analysis, which describes the total variation inherent in the data, identified the variables PRO_1 and PRO_2 (Table 3) with factor loadings greater than 0.7. DFA was more useful since it identified the variables that were sufficiently variable to discriminate between taxa, even though they had a relatively small contribution towards the overall observed variation within the whole data set. The means and standard deviations for each of the taxa considered demonstrate discrimination between some of the taxa on the basis of a few variables (Figure 4). ANOVA for each of the variables indicate that the differences between taxa are highly significant (Figure 4). The *post hoc* comparisons indicate which variables enable discrimination between taxonomic group pairs and allow us to consider the relationships of those groups. There are significant differences between the C-A means of *T. monteiri* and each of the subspecies of Redbilled Hornbill. The C-A means are also significantly different between *T. e. kempi* and both *T. e. damarensis* and *T. e. rufirostris* respectively. However, the range of C-A values for *T. e. damarensis* and *T. e. rufirostris* overlap with that of *T. monteiri* (Figure 4). Similarly, the range of C-A values for both *T. e. damarensis* and *T. e. rufirostris* overlaps with that of *T. e. kempi*. The second variable C_RATE2 also has significant different means between both *T. e. erythrorhynchus* calls and *T. e. kempi* calls and each of the other taxonomic groups respectively. However, there is also overlap of the ranges of values for C_RATE2 (Figure 4). Finally B-D shows an increase in values with the order in which the taxa occur in the figure. There are two clusters of observations for B-D: the first cluster includes the taxa with low B-D values

**Table 2** : Matrices of percentage correct classification achieved in forward discriminant function analysis of *Tockus monteiri* and the *Tockus erythrorhynchus* subspecies. Percentage correct classifications between taxa are above the diagonal and sample sizes are below the diagonal.

| a) | 1. | 2. | 3. | 4. | 5. |
|---|---|---|---|---|---|
| 1. *T. e. erythrorhynchus* | | 95.65 | 100 | 100 | 100 |
| 2. *T. e. rufirostris* | 23 | | 79.45 | 100 | 100 |
| 3. *T. e. damarensis* | 64 | 73 | | 100 | 100 |
| 4. *T. e. kempi* | 15 | 24 | 65 | | 100 |
| 5. *T. monteiri* | 11 | 20 | 61 | 12 | |

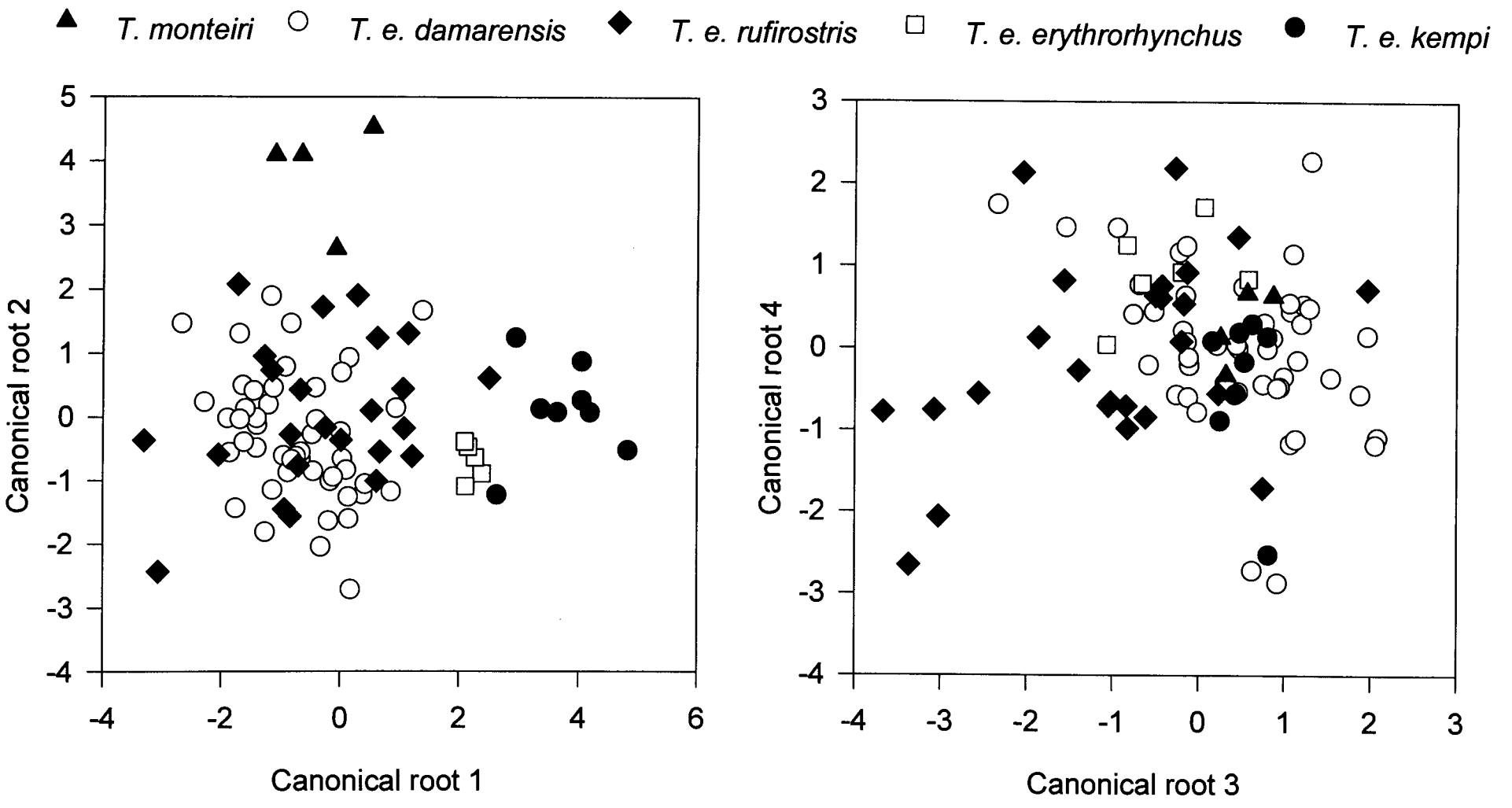



**Figure 3**: Scatterplot of the canonical roots from forward Discriminant Function Analysis of sound recordings of calls from all the subspecies of Redbilled Hornbill and Monteiro's Hornbill.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

**Table 3**: Results from Principal Component Analysis (PCA) and Discriminant Function Analysis of the twelve call variables for the five taxonomic groups. PC1 = principal component 1, CPCS = cumulative percentage classification success and P = probability value. Variables included in the *a posteriori* univariate analysis are indicated in bold.

| **Variable** | **PCA** | **Discriminant Function Analysis** | | |
|---|---|---|---|---|
| | **PC1 loading** | **CPCS** | **Wilk's Lamba** | **P** |
| **B-D** | -0.211 | 68.48 | 0.185 | <0.001 |
| **C_RATE2** | 0.173 | 70.65 | 0.136 | <0.001 |
| **C_A** | 0.099 | 72.83 | 0.153 | <0.001 |
| EM_FREQ | 0.097 | 81.52 | 0.140 | 0.015 |
| N_C1 | 0.582 | 81.52 | 0.136 | 0.016 |
| MDUR_C1 | -0.027 | 81.52 | 0.129 | <0.001 |
| DUR | 0.124 | 80.43 | 0.157 | 0.072 |
| N_C2 | -0.576 | 82.61 | 0.158 | 0.196 |
| PRO_1 | 0.975 | 83.69 | 0.138 | 0.159 |
| C_RATE1 | -0.058 | 81.52 | 0.133 | 0.049 |
| PRO_2 | -0.926 | 82.61 | 0.130 | 0.292 |
| MDUR_BC1 | 0.029 | Not included in f-DFA model | | |

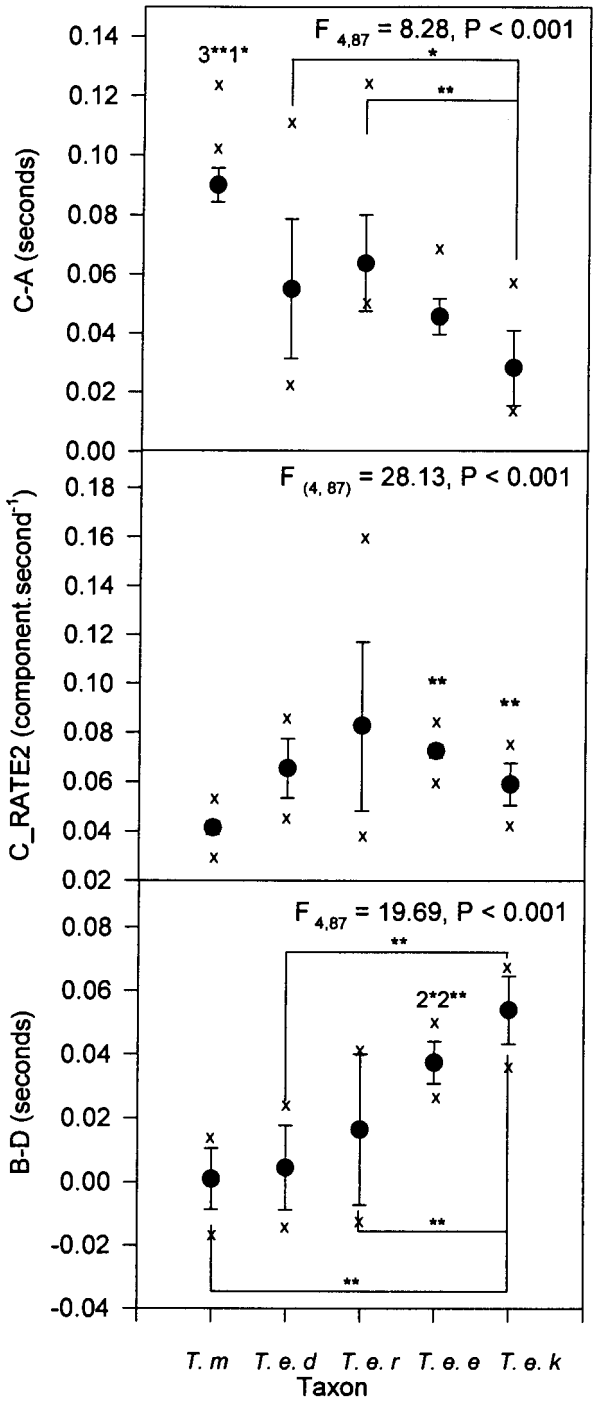



**Figure 4**: Taxon-specific variable means and standard errors for the top three discriminating variables of the forward Discriminant Function Analyses. ANOVA results for each variable are presented, as are the results of the *post hoc* comparisons of means. Levels of significance are indicated by * (95%), ** (99%) and ns (not significant). Where indications appear above the error bars the significance or non-significance refers to that taxon compared with all others whereas lines joining a pair of taxa refer to the significance or non-significance of the particular taxon pair. The variables' ranges are represented by x at the upper and lower extremes for each taxonomic group.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

(*T. monteiri*, *T. e. damarensis* and *T. e. rufirostris*) while the second cluster includes the taxa with higher B-D values (*T. e. erythrorhynchus* and *T. e. kempi*). Most significant *post hoc* comparisons occur between members of these two clusters and a t-test comparing the means for the two clusters yields a highly significant difference ($T = -7.67$, $p << 0.001$, $df = 90$). The variable B-D shows the least overlap of ranges between the two groups of taxa (group 1: *T. monteiri*, *T. e. damarensis*, *T. e. rufirostris*; group 2: *T. e. erythrorhynchus* and *T. e. kempi*) that had significantly different means in an ANOVA. Only *T. e. rufirostris* has a range of values that encompasses calls with both small and large B-D values.

**Discussion**

*Differences between vocalisations of Tockus hornbills*

Inspection of sonograms prior to statistical analysis suggested that the different Redbilled Hornbill taxa have constant differences in the double-clucks of the second escalated component of their courtship call (Figure 1, Figure 2). *Tockus monteiri* and *T. e. damarensis* have a reduction in the time interval between the first and second notes of the double-clucks, relative to the time interval between double-clucks. *Tockus e. kempi* individuals have double-clucks with a reduction in the duration of the second note relative to the first. *Tockus e. rufirostris* and *T. e. erythrorhynchus*, however, show both of the former characteristics. We were able to make a number of predictions regarding the variables C-A and B-D according to the characteristics of the double-clucks. First *T. monteiri* and *T. e. damarensis*, which only have reduction in the time interval between successive notes of double-clucks, should have the highest values for C-A, while *T. e. kempi* individuals should have the lowest values for C-A. *Tockus e. rufirostris* and *T. e. erythrorhynchus*, which have both characteristics, should also have C-A values greater than that of *T. e. kempi*. Second, *T. monteiri* and *T. e. damarensis* should have low values for B-D, since they don't have double-clucks with a reduction in the duration of the second note relative to the first. The taxa that have such a reduction (*T. e. rufirostris, T. e. erythrorhynchus* and *T. e. kempi*) should have larger values for the variable B-D. The results (Figure 4) support these predictions except for two anomalies. First, calls of *T. e. damarensis* do not exhibit C-A values as large as those of the *T. monteiri* calls and, second, calls of *T. e. rufirostris* do not have large B-D values. In addition, the clusters of observations



15779828
b152 31781

for *T. e. rufirostris* and *T. e. damarensis* overlap in the scatterplot of canonical roots one and two (Figure 3). The calls of these subspecies were recorded both in areas of sympatry and in allopatry. We observed *T. e. damarensis – T. e. rufirostris* heterospecific pairs in the contact zone in northern Namibia (unpublished data) which suggests the two subspecies hybridise in that area. In many nonpasserine birds the calls of hybrids are intermediates between the parental species (Payne 1986), attributed to the genetic inheritance of vocalisation. Therefore, the two anomalies may be attributed to hybridisation since the *T. e. damarensis* calls have C-A values closer to those of *T. e. rufirostris* than expected and the *T. e. rufirostris* calls have B-D values closer to those of *T. e. damarensis* than expected.

*Univariate vs Multivariate analysis*

The ranges of the three variables most useful in discriminating between the five *Tockus* taxa provide an indication of whether univariate or multivariate analysis is more appropriate. The univariate analysis identified significantly different means between the taxa considered. However, since the variables' ranges of the taxa overlap they are unreliable when identifying species specific vocalisations. The variable B-D is the most robust in its discriminatory power as its ranges are divided into two clear taxonomic groups. The first encompasses species that have no differences in the duration of the first and second notes of the double-clucks (*T. monteiri*, *T. e. damarensis* and *T. e. rufirostris*), whilst the second group includes species that do have such difference (*T. e. erythrorhynchus* and *T. e. kempi*). The *T. e. rufirostris* values that encompass the range of both groups is probably attributed to hybridisation. An analysis of the change of call pattern across the *T. e. damarensis* - *T. e. rufirostris* hybrid zone is therefore planned for future work.

*Species concepts and implications*

The requirement that animals need consistent diagnosable traits to be identified as belonging to different species is common to all species concepts including the Recognition Species Concept (Paterson 1985), Biological Species Concept (Mayr 1963, Dobzhansky 1970), Phylogenetic Species Concept (Cracraft 1989) and Cohesion Species Concept (Templeton 1989). The calls of at least two subspecies of Redbilled Hornbill (*T. e. damarensis* and *T. e. rufirostris*) are consistent between allopatric populations that cover the geographic ranges of these subspecies. This result

suggests that the call differences between the subspecies do not occur at the population level but rather at the level of subspecies. Therefore, diagnosable differences in the morphological and behavioural traits above suggest an investigation into the specific status for each of the subspecies of Redbilled Hornbill is required. This is not because differences in mate recognition characteristics are consistent and diagnosable in the operational sense (Sokal 1974), but because it could reflect the process of speciation within this group. Furthermore, an analysis of gene flow between the divergent populations of Redbilled Hornbill would lend support to our argument for specific status.

*Phylogenetic relationships among Redbilled Hornbills*

The use of behavioural traits to infer the relationships of animal taxa has gained much support in recent years (McLennan *et al.* 1988, Page & Johnston 1990, Prum 1990, Langtimm & Dewsbury 1991, de Queiroz & Wimberger 1993, Paterson, Sullivan & Malmos 1994, Wallis & Gray 1995). Lanyon (1969) concluded that avian vocal characters hold much promise in the fields of avian systematics and evolutionary biology and vocal characters that serve in species recognition, mate selection and courtship would have the most promising role. However, there is conflict between the morphological and behavioural data sets considered. Our preliminary analysis of sonograms suggested that the vocalisations of *T. e. rufirostris* are more similar to *T. e. erythrorhynchus* than either is to *T. e. damarensis* (Figure 1). Although the quantitative analysis of vocalisations suggested the calls of *T. e. damarensis* and *T. e. rufirostris* were more closely related, this anomaly is probably due to hybridisation. Morphological characters, on the other hand, indicate that there may be two phylogenetic groups; (i) a northern group (*T. e. erythrorhynchus*, *T. e. kempi* and the northern Kenyan and southern Tanzanian populations) that comprise populations with black facial skin, suborbital or circumorbtial, and (ii) a southern group (*T. e. rufirostris* and *T. e. damarensis*) with only pink circumorbital skin. The Yellow-billed Hornbill species group is divided into two species, *T. leucomelas* the southern Yellow-billed Hornbill with pink circumorbital skin and *T. flavirostris* the Eastern Yellow-billed Hornbill with black circumorbital skin (Kemp 1995). Although Yellow-billed Hornbills do not occur in West Africa, their southern and eastern populations have similar geographic distributions to the Redbilled Hornbills (Kemp 1995). Therefore, the Redbilled Hornbills may similarly be divided into two principal

phylogenetic lineages that comprise divergent populations. Finally the courtship display behaviour of Redbilled Hornbills further complicate the taxonomic relationships. *T. e. damarensis* and *T. e. erythrorhynchus* display with their wings partially open (Figure 1), which suggests they are more closely related to each other than to *T. e. rufirostris* that display with closed wings. We intend resolving the phylogenetic relationships among the six known geographical variants of the African Redbilled Hornbills using recordings of the northern Kenyan and southern Tanzanian Redbilled Hornbill populations, combined with morphological, behavioural and DNA sequence data.

# Chapter 3

## The structure of an African Redbilled Hornbill hybrid zone revealed by morphology, behaviour and breeding biology

"A question has arisen ...concerning the manner in which their intergradation is accomplished. Is one bird an imperfectly differentiated offshoot of the other, and are the connecting intergrades geographical intermediates, or have we here two distinct species whose intergradation is due to interbreeding where the confines of their respective habitats adjoin?"

Chapman 1892

**Abstract**

The southern African (*Tockus erythrorhynchus rufirostris*) and Damaraland Redbilled Hornbills (*Tockus erythrorhynchus damarensis*) have distributions that overlap in northern Namibia. Allopatric populations of these subspecies exhibit diagnosable differences in habitat, morphology, vocalisation and displays. We investigated the structure of the hybrid zone using morphological, behavioural and breeding data. The morphological characteristics, eye colour and facial plumage colour, were summarised as hybrid index scores. These hybrid index scores exhibited a significant positive regression against distance from southwest to northeast across the hybrid zone. Although intermediate phenotypes are present further northeast than southwest in the contact zone, this spatial asymmetry may be the result of asymmetric dispersal. Vocalisations also showed a positive relationship between the first principal component (extracted from 12 call variables) and distance across the hybrid zone. In addition, there appears introgression of a *T. e. damarensis* call into *T. e. rufirostris* but not *vice versa*. Such asymmetric call introgression may be the result of a number of processes. However, in this case we limit it to either asymmetry in assortative mating or the genetic control of call inheritance. Moreover, female *T. e. damarensis* - male *T. e. rufirostris* hybrid pairs occur more frequently than the alternative combination. Finally, heterospecific pairs do show lower fitness in the form of reduced hatching success even when female fitness attributes are included as covariates. We conclude that the Redbilled Hornbill hybrid zone resembles both the Mosaic and Tension Zone models since it is determined by both habitat characteristics and a balance of dispersal and selection.

## Introduction

Hybridisation has long fascinated ornithologists and continues to retain a prominent role in ornithological research. Such research can address a multitude of phenomena with some authors (Hewitt 1988, Barton & Hewitt 1989, Rohwer & Wood 1998) describing hybrid zones as natural laboratories for evolutionary and population biology. Similarly, mathematical models of hybrid zones have shown that they are conducive towards a better understanding of selection, dispersal, competition, mate choice and the nature of the genetic control of phenotypic characters (Endler 1977, Barton 1979, Barton & Hewitt 1985, 1989, Barton & Gale 1993, Gill 1997). A primary aim of hybridisation studies has been to identify the processes occurring within zones of overlap and thereby contributing towards a general hybrid zone theory. However, these processes vary between cases since the context in which hybrid zones occur differs (Rand & Harrison 1989). Three hybrid zone models proliferate in the literature. The Tension Zone model depends on a balance between dispersal and selection against hybrids (Barton & Hewitt 1985). The Hybrid Superiority Model requires that hybrids be selected for within a transition between habitats (Moore 1987, Arnold & Hodges 1995). Finally, the Mosaic Model is dependent on a mosaic of habitat types that causes a mosaic distribution of taxa with alternate habitat preferences (Harrison & Rand 1989, Rand & Harrison 1989).

Two subspecies of African Redbilled Hornbill (*Tockus erythrorhynchus rufirostris* and *Tockus erythrorhynchus damarensis*) hybridise in a contact zone in northern Namibia. The Damaraland Redbilled Hornbill (*T. e. damarensis*) is endemic to Namibia, whilst the southern African Redbilled Hornbill (*T. e. rufirostris*) is common to the savanna woodlands of southern Africa (Malawi, Luangwa and Zambezi valleys in Zambia, Zimbabwe, northern Botswana, southern Angola, northeastern Namibia, southeastern Mozambique and northeastern South Africa). These two subspecies differ in morphology (Kemp 1995), courtship vocalisations (Delport 2000a Chapter 2) and courtship displays (Kemp 1995). *Tockus e. damarensis* have brown eyes and white facial plumage whereas *T. e. rufirostris* have yellow eyes and grey facial plumage (Figure 1). Furthermore,

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA


**Figure 1:** Variation in facial plumage and eye colours of *T. e. rufirostris*, *T. e. damarensis* and hybrid Redbilled Hornbills. A = allopatric *T. e. damarensis* with white facial plumage and brown eye, B = hybrid with intermediate facial plumage and brown eye, C = hybrid with grey facial plumage and brown-yellow eye, D = hybrid with grey facial plumage and yellow-brown eye and E = allopatric *T. e. rufirostris* with grey facial plumage and yellow

hybrids appear to have an intermediate facial plumage (Sanft 1960) and eye colour (Figure 1). The vocalisations of Redbilled Hornbills are a series of clucking notes that break into double clucking notes (Kemp 1995, Delport 2000a Chapter 2, Figure 2). Delport (2000a Chapter 2) has demonstrated that there are differences between the vocalisations of four subspecies of Redbilled Hornbill (including *T. e. damarensis* and *T. e rufirostris*) in how the double-clucking notes of the vocalisations are achieved. Finally, the courtship display of Redbilled Hornbills, that occurs during vocalisation, comprises a bobbing motion of the body with the head held down. During this display the wings may be held fully open, partially open or closed depending on the subspecies (Kemp 1995). We address hybridisation between *T. e. damarensis* and *T. e. rufirostris* using morphological, behavioural and breeding data. Thereafter, we determine which of the hybrid zone models best characterises the Redbilled Hornbill hybrid zone.

## Methods and Materials

*Morphological data collection and analysis*

The study area was in the Otavi mountain region (19° 20' S, 17° 21' E), northern Namibia, where the distributions of *T. e. damarensis* and *T. e. rufirostris* overlap. The purpose of the morphological and morphometric analysis was to (i) represent change of phenotypic characteristics across a contact zone and (ii) to determine if allopatric populations of both subspecies were diagnosable as distinct. We therefore collected geographic distribution data of *T.e damarensis*, *T. e. rufirostris* and hybrids across the contact zone and scored individuals according to phenotypic traits that are easy to identify with binoculars (Table 1). Hybrid indices were calculated for each individual on the basis of these phenotypic traits (Table 1). We grouped observations into 13 localities and mapped hybrid index histograms (Figure 3). In addition to the morphological hybrid index we measured bill length, bill width and tarsus length (to the nearest 0.1mm using a vernier calliper) from allopatric and sympatric males and females of both subspecies. Nine sympatric *T. e. damarensis* females, 15 sympatric *T. e. rufirostris* females, 23 sympatric hybrid females and 3 sympatric *T. e. rufirostris* males were measured from the

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

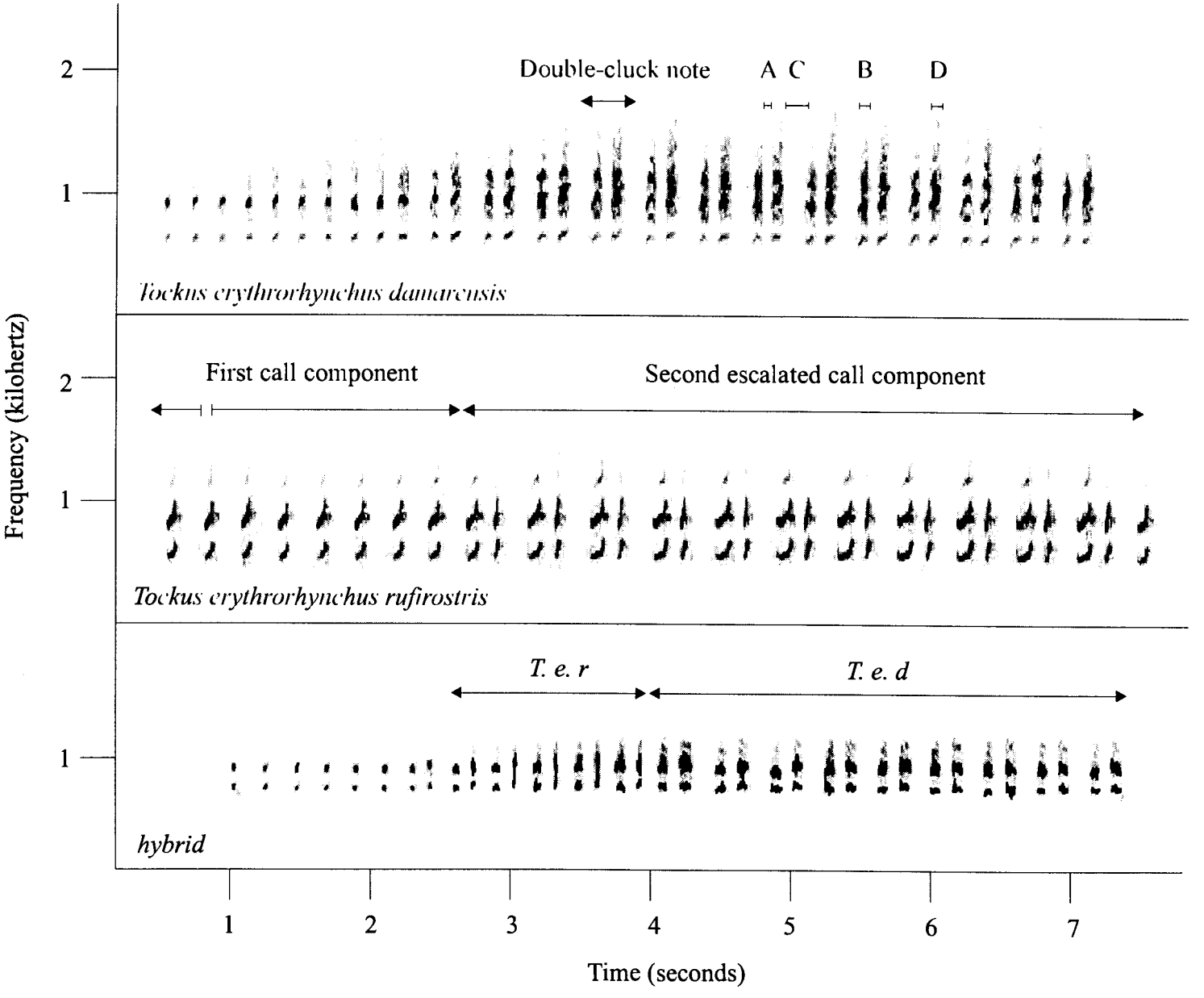



**Figure 2:** Spectrograms of the courtship calls of the two subspecies of the African Redbilled Hornbills and one hybrid. The call terminology used is represented, as are some of the variables used in the multivariate analyses. The first call component arrow line is broken since the call starts earlier than represented.

**Table 1:** Combinations of phenotypic characters that constitute each of the phenotypic hybrid index scores used in the morphological analyses. Hybrid index = eye score + plumage score.

| Hybrid Index | Eye colour | Eye score | Facial plumage colour | Plumage score | # Birds Observed |
|---|---|---|---|---|---|
| 2 | Brown | 1 | White | 1 | 54 |
| 3 | Brown | 1 | White-grey | 2 | 10 |
| | Brown-yellow | 2 | White | 1 | 10 |
| 4 | Brown | 1 | Grey | 3 | 63 |
| | Brown-yellow | 2 | White-grey | 2 | 5 |
| 5 | Brown-yellow | 2 | Grey | 3 | 16 |
| | Yellow-brown | 3 | White-grey | 2 | 12 |
| | yellow | 4 | White | 1 | 0 |
| 6 | Yellow-brown | 3 | Grey | 3 | 21 |
| | Yellow | 4 | White-grey | 2 | 0 |
| 7 | Yellow | 4 | Grey | 3 | 202 |

contact zone in northern Namibia. Ten allopatric *T. e. damarensis* females from Daan Viljoen Game Reserve near Windhoek, Namibia (22° 32' S, 16° 58' E) were measured as well as 13 allopatric *T. e. damarensis* males from the collection of skins at the State Museum, Windhoek, Namibia. Fifteen allopatric *T. e. rufirostris* females and 25 allopatric *T. e. rufirostris* males were measured from the collection of skins at the Transvaal Museum, Pretoria, South Africa. These measurements were included in a Principal Component Analysis. Thereafter, we performed an ANOVA and *post hoc* comparison of means (Duncan's Multiple Range test) on the values of the first principal component for sympatric and allopatric individuals of both subspecies and hybrids. In addition to the PCA, we performed a Discriminant Function Analysis of allopatric morphometrics of males and females combined and independently. The Discriminant Function Analysis (i) identified which variables are useful for discrimination between groups and (ii) gave an indication on how reliable the morphometric discrimination between the subspecies were.

*Sound acquisition*

We recorded 201 display vocalisations from 85 individual male Redbilled Hornbills across the contact zone, from phenotypically pure *T. e. damarensis* in the southwest to phenotypically pure *T. e. rufirostris* in the northeast. Only males, which are distinguished from females by the black colouration of the lower bill (Kemp 1995), were recorded during the breeding seasons of 1999 (15 January - 1 April) and 2000 (1 February - 15 March) since females are sealed within a nest cavity during this period. We used a Sony TCM-38V cassette recorder with a Tect 913 directional microphone during the 1999 breeding season and a Tascam DAP-1 DAT field recorder with a Nakamichi CM-300 microphone and Sony PBR-330 parabolic reflector during 2000. In addition to the above calls from the contact zone, we included calls from allopatric populations of both subspecies. We obtained the calls of eight *T. e. rufirostris* individuals, recorded in South Africa, from the Fitzpatrick Sound Communication Library, Transvaal Museum, Pretoria, South Africa. Allopatric calls of *T. e. damarensis* (n = 11) were recorded at Daan Viljoen Nature Reserve near Windhoek, Namibia, using the above equipment. We included these allopatric calls in the analyses to (i) compare pure allopatric *T. e. rufirostris* and *T. e.*

*damarensis* calls with those recorded at the northeastern and southwestern extremes of the contact zone respectively, and (ii) to identify the variables that best discriminate between allopatric populations of both subspecies.

*Sonographic analyses*

The courtship call of Redbilled Hornbills consists of a series of single-clucking notes that accelerate and break into double-clucking notes (Figure 2). We refer to the first part of the call as the first call component and the part after the double-clucking notes begin as the second escalated component (Delport 2000a Chapter 2). Hereafter, single-clucking notes and double-clucking notes will be referred to as single clucks and double clucks respectively. The calls recorded were digitised (at a sampling frequency of 6000 Hz) on a personal computer using Avisoft-Sonograph Pro for Windows version 2.75. We generated two types of sonograms for analysis. First, fine temporal scale sonograms were used when we measured short duration, i.e. duration of clucks and between clucks. Second, coarse temporal scale sonograms were used when we measured long duration, i.e. duration of the entire call and its first and second components. Coarse temporal scale sonograms were used for the long duration measurements since we could visualise the entire call on the computer screen, whereas only short sections of the call could be visualised in the fine temporal scale analysis. The coarse temporal scale sonograms were also used when frequency values were measured. Fine temporal scale sonograms were generated using the following parameter values, FFT = 128, Frame (%) = 100, Window = hamming, Overlap = 93.75. The analysis resolutions of the fine scale sonograms were 1.33 ms and 60 Hz for time and frequency respectively. Coarse temporal scale sonograms were generated using the following parameter values, FFT = 256, Frame (%) = 100, Window = hamming, Overlap = 50.0, that resulted in analysis resolutions of 21.33 ms and 30 Hz for time and frequency respectively. Delport (2000a Chapter 2) measured twelve call variables from sonograms of four of the subspecies of African Redbilled Hornbill. We measured these same twelve variables (Table 2, Figure 2) in our analyses of the calls from a contact zone between *T. e. damarensis* and *T. e. rufirostris*.

**Table 2**: Variables measured from sonograms and used in statistical analysis of calls across a contact zone between *T. e. damarensis* and *T. e. rufirostris*. Mean values and standard deviations (in parenthesis) are represented (a-*T.e.d* = allopatric *Tockus erythrorhynchus damarensis*, s-*T.e d* = *sympatric Tockus erythrorhynchus damarensis*, s-*T.e.r* = sympatric *Tockus erythrorhynchus rufirostris* and a-*T.e.r* = allopatric *Tockus erythrorhynchus rufirostris*).

| Mnemonic | Variable | Units | Taxon mean (standard deviation) | | | |
|---|---|---|---|---|---|---|
| | | | *a-T.e.d* | *s-T.e.d* | *s-T.e.r* | *a-T.e.r* |
| DUR | Total duration of call | Seconds | 9.8 (2.6) | 8.9 (1.52) | 9.4 (2.13) | 10.3 (3.9) |
| PRO_1 | Proportion of call comprising the first component | % | 46.7 (8.9) | 43.9 (10.3) | 41.1 (11.1) | 40.9 (10.0) |
| PRO_2 | Proportion of call comprising the second escalated component | % | 53.1 (8.9) | 56.2 (10.3) | 58.9 (11.0) | 59.0 (9.9) |
| MDUR_C1 | Mean duration of all the notes in the first call component | Seconds | 0.054 (0.008) | 0.047 (0.006) | 0.049 (0.011) | 0.057 (0.011) |
| MDUR_BC1 | Mean duration between notes in the first call component | Seconds | 0.172 (0.035) | 0.165 (0.025) | 0.157 (0.020) | 0.152 (0.039) |
| N_C1 | Number of notes in first call component | # | 20.2 (6.8) | 18.4 (8.1) | 18.6 (8.0) | 19.6 (13.3) |
| N_C2 | Number of notes in the second escalated call component | # | 32.0 (10.8) | 31.2 (6.5) | 35.9 (9.8) | 40.3 (10.3) |
| A* | Mean duration within double-clucks | Seconds | 0.057 (0.016) | 0.060 (0.018) | 0.055 (0.014) | 0.054 (0.015) |
| B* | Mean duration of the first notes of double-clucks | Seconds | 0.079 (0.007) | 0.076 (0.011) | 0.076 (0.022) | 0.077 (0.009) |
| C* | Mean duration between double-clucks | Seconds | 0.126 (0.018) | 0.124 (0.016) | 0.127 (0.019) | 0.116 (0.023) |
| D* | Mean duration of the second notes of double-clucks | Seconds | 0.083 (0.011) | 0.072 (0.016) | 0.064 (0.021) | 0.045 (0.006) |
| C – A | The duration mean A subtracted from the duration mean C | Seconds | 0.064 (0.019) | 0.064 (0.020) | 0.065 (0.020) | 0.059 (0.020) |
| B – D | The duration mean D subtracted from the duration mean B | Seconds | -0.003 (0.011) | 0.003 (0.014) | 0.016 (0.025) | 0.034 (0.011) |
| C_RATE1 | Rate at which notes are uttered in the first call component | clucks/second | 4.4 (0.7) | 4.5 (0.7) | 4.7 (0.5) | 4.4 (0.5) |
| C_RATE2 | Rate at which notes are uttered in the second escalated call component | clucks/second | 6.1 (0.7) | 6.3 (0.7) | 6.5 (0.8) | 6.9 (1.1) |
| EM_FREQ | Emphasized frequency (frequency emmited at the loudest amplitude) of the call | Hertz | 1066.5 (67.7) | 1036.1 (73.1) | 1006.6 (80.6) | 1107.8 (143.3) |

* Measurements that were not included in the analyses, but were used to calculate some variables.

*Statistical analyses of calls*

The statistical analyses of calls aimed to achieve three goals, firstly to identify the variables most useful for discriminating between subspecies, secondly to compare calls from allopatric populations of both subspecies, and thirdly to represent the variation of calls across the contact zone. In the first analysis, we performed a Principal Component Analysis (PCA) of 12 variables from calls of allopatric populations of *T. e. damarensis* and *T. e. rufirostris*, from Windhoek and South Africa respectively. We also performed a forward stepwise Discriminant Function Analysis (DFA) using the same data. PCA quantifies all the variation inherent in the data whilst Discriminant Function Analysis only identifies that part of the variation useful for discriminating between groups. The most useful discriminating variables were identified as those included in the DFA model with $F > 1.0$.

We then included the most useful discriminating variables in a PCA of calls from allopatric populations of both subspecies (*T. e. damarensis* n = 11, *T. e. rufirostris* n = 8) and from the northeastern (*T. e. rufirostris* n = 3) and southwestern (*T. e. damarensis* n = 11) extremes of the contact zone. The aim of this second analysis was (i) to determine if calls from allopatric populations of both subspecies were distinct and (ii) to determine if calls from the northeastern and southwestern extremes of the contact zone differed from their allopatric counterparts. We followed this up with a one-way ANOVA of the first principal component values and a Duncan's Multiple Range test of means.

In the third analysis, of the change of call structure across the contact zone, we included the most useful discriminating variables in a PCA of calls recorded across the contact zone (n = 85) as well as those from allopatric populations of both subspecies (*T. e. damarensis* n = 11, *T. e. rufirostris* n = 8). Thereafter, we grouped the 85 contact zone calls, into the same thirteen localities used in the morphological analysis, and mapped histograms of the first principal component (PC1) for calls from each locality. Typical *T. e. damarensis* and *T. e. rufirostris* calls were identified as those with PC1 values within the range of allopatric *T. e. damarensis* and *T. e. rufirostris* calls respectively. In addition, we executed a linear regression of distance (across the contact zone) with the first

principal component values. Finally, we aimed to determine whether there were differences in the degree to which each call type introgressed in the hybrid zone and plotted the PC1 values against distance from the proposed centre of the contact zone. The centre of the contact zone is the region where we observed equal proportions of *T. e. rufirostris* and *T. e. damarensis* as well as a majority of hybrid phenotypes.

*Breeding pair composition*

Distribution data of homo- and heterospecific breeding pairs were collected from the central 100km of the contact zone. We only used data from the central 100km since only here would *T. e. damarensis* and *T. e. rufirostris* individuals have approximately equal opportunities to mate with members of either subspecies. Males and females of each pair were scored phenotypically according to the hybrid index previously discussed. However, for the analysis of pair formation that follows we only differentiated between *T. e. damarensis*, *T. e. rufirostris* and hybrids. All birds with brown eyes were lumped as *T. e. damarensis*; those with yellow eyes as *T. e. rufirostris* and those with intermediary eye colours as hybrids. We used eye colour over facial plumage colour as an indicator of subspecies and hybrids since it is less subjective to score than the latter. The data were simplified in this manner since the breeding pairs sample size of 117, was too small to differentiate between the seven classes (which would yield 49 breeding pair combinations) used in the phenotypic hybrid index. We proceeded with an exact test for randomness of mating (Haldane 1954 modified by Louis & Dempster 1987), which gives the exact probability of drawing the observed breeding pair combinations from a random-mating population. We calculated a binomial probability of heterospecific mating, which indicated whether there was an asymmetrical relationship of hybridisation towards a particular pair combination.

*Breeding biology data collection and analysis*

Although hornbills normally nest in tree cavities (Kemp 1976, 1995), they also breed in artificial nestboxes (Riekert & Clinning 1985). We distributed 200 breeding boxes (495 mm x 200 mm x 240 mm; entrance diameter = 52 mm) across four farms in the contact zone, moving from predominantly *T. e. rufirostris* in the northeast to predominantly *T. e.*

*damarensis* in the southwest. During the 1999 and 2000 breeding seasons, February – March, we periodically checked nestboxes for occupation by females. For each occupied nestbox, we recorded subspecies of both sexes of the breeding pair, female mass after laying, female morphometrics, egg volume, clutch size and brood size (see below).

Female mass. We used Salter 0-100g and 0-200g balances and a Pesola 0-300g balance to weigh females in a canvas bag. The mass of the canvas bag was determined immediately after each weighing. Females were weighed four to five days after they had laid the last egg of their clutch. Redbilled Hornbills lay their eggs approximately two days apart and therefore it was necessary to wait four to five days to establish that the clutch was complete. We only weighed females after they had laid their complete clutch since they may desert if handled before (W. Delport – unpublished data).

Female morphometrics and egg volume. Bill width, bill length and tarsus length were measured (to the nearest 0.1 mm) of females in hand using a Vernier caliper. Egg breadth and width were measured for each egg of each clutch and we used Hoyt's (1979) formula (egg volume = eggshell length x breadth$^2$ x 0.51) to calculate egg volume.

Clutch size and brood size. Clutch size was determined as the number of eggs five days after the last egg was laid. We monitored the nestboxes during the breeding period and in no instance was an egg laid later than five days after the former (W. Delport – unpublished data). Brood size was determined after 30 days of incubation of the last egg of the clutch. The mean incubation period for Redbilled Hornbills is 24 days (Kemp 1995), and therefore it was assumed that eggs that had not hatched after 30 days were never going to hatch. We opened the eggs that had not hatched and noted their stage of development.

*Statistical analyses of breeding biology data*

The statistical tests we conducted were intended to (i) identify if there were differences in breeding success between homo- and heterospecific pairs and (ii) to determine if any of these differences were related to female fitness attributes. We first performed an ANOVA on three measures of breeding success (clutch size, brood size and hatching success) for nineteen homo- and nineteen heterospecific breeding pairs. Deleterious effects of

hybridisation could manifest at any stage of the life cycle, however they are most likely to affect the early development or fertility of hybrid phenotypes (Moore & Koenig 1986). Hatching success was included since incompatibility between subspecies may be evident at fertilisation. Fledgling success was not analysed since it may have an environmental component, especially when comparing data from different breeding seasons. Secondly, we performed an ANCOVA of the measures of breeding success that indicated significant differences between homo- and heterospecific breeding pairs. Five female fitness attributes (mass after laying, bill length, bill width, tarsus, mean egg volume) were used as covariates to determine if any of these were significantly related to the differences in breeding success between homo- and heterospecific pairs.

**Results**

*Morphology and morphometrics*

The distribution of phenotypes across the contact zone represents a cline from pure *T. e. damarensis* in the southwest to predominantly pure *T. e. rufirostris* in the northeast (Figure 3). This was clear from a regression analysis of location against hybrid index that yielded a significant positive relationship ($r^2 = 0.277$, $F_{1, 370} = 143.09$, $p < 0.001$) across the contact zone. West of Outjo the phenotypes are typically *T. e. damarensis* with intermediates occurring between Outjo and Tsumeb. Northeast of Tsumeb and Grootfontein *T. e. rufirostris* phenotypes proliferate. However it appears that more *T. e. rufirostris* phenotypes penetrate into the contact zone than do the corresponding *T. e. damarensis* phenotypes. Localities 7 and 8 (Figure 3) have higher proportions of pure *T. e. rufirostris* than pure *T. e. damarensis* phenotypes even though these localities are relatively central between the *T. e. damarensis* and *T. e. rufirostris* phenotype extremes of the contact zone (localities 4, 5 and 12, 13 respectively). In addition, the habitats of the northeastern and southwestern extremes of the contact zone have different abundances of Redbilled Hornbills (southwest thornveld = 1 bird every 12.1 km, central Otavi mountain region = 1 bird every 3.8km, northeast woodland = 1 bird every 6.2km). Furthermore, hybrid phenotypes occur further northeast than southwest (localities 10, 11, 12 & 13). These hybrid phenotypes that occur northeast of Otavi comprise a range of hybrid index

scores (3-6) which represent phenotypic characteristics of both *T. e. damarensis* and *T. e. rufirostris* (Table 1). In the morphometric analysis, the ANOVA of the first principal component (PC1) revealed significant differences between allopatric males and females of the two subspecies (Figure 4). Sympatric representatives of both subspecies and hybrids have intermediate PC1 values in both males and females (Figure 4). Furthermore the Principal Component Analysis indicated that all three variables were highly variable (since they had factor loadings greater than 0.7), and were therefore included in the first principal component. Discriminant Function Analysis, however, indicated that bill length is the most reliable for discriminating between groups (BL: $F_{3, 57} = 13.94$, $P < 0.001$; BW: $F_{3, 57} = 3.39$, $P < 0.05$; TAR: $F_{3, 57} = 2.39$, $P > 0.05$). The classification success of allopatric males and females to their respective subspecies was 77%, which increased when sexes were analysed separately (male: 81.5%; female: 96%).

*Statistical analyses of calls*

Discriminating call variables. The Principal Component Analysis indicated that the variables PRO_1, PRO_2, MDUR_BC1, N_C2 and C_RATE1 explained a large amount of the variation inherent in the call data, since they had factor loadings greater than 0.7 (Table 3a). However Discriminant Function Analysis only included three variables in the forward stepwise model: B-D, MDUR_BC1 and EM_FREQ (Table 3b). The contribution of variable B-D to the discrimination between the subspecies is greater than that of the other two variables (MDUR_BC1 and EM_FREQ) included in the Discriminant Function Analysis model. The variables that were utilised in the subsequent Principal Component Analyses were B-D, MDUR_BC1 and EM_FREQ.

Comparison of allopatric calls. The ANOVA of the first principal component values of allopatric calls of both subspecies (and from the southwestern and northeastern extremes of the contact zone) yielded a highly significant result ($F_{3,29} = 18.97$, $p << 0.001$). The Duncan's Multiple Range test identified that significant differences occurred between all groups except between the allopatric populations and their southwestern and northeastern contact zone counterparts (Table 4).

Change of calls across the contact zone. The PCA that included allopatric populations of both subspecies and individuals recorded in the contact zone served two purposes. The

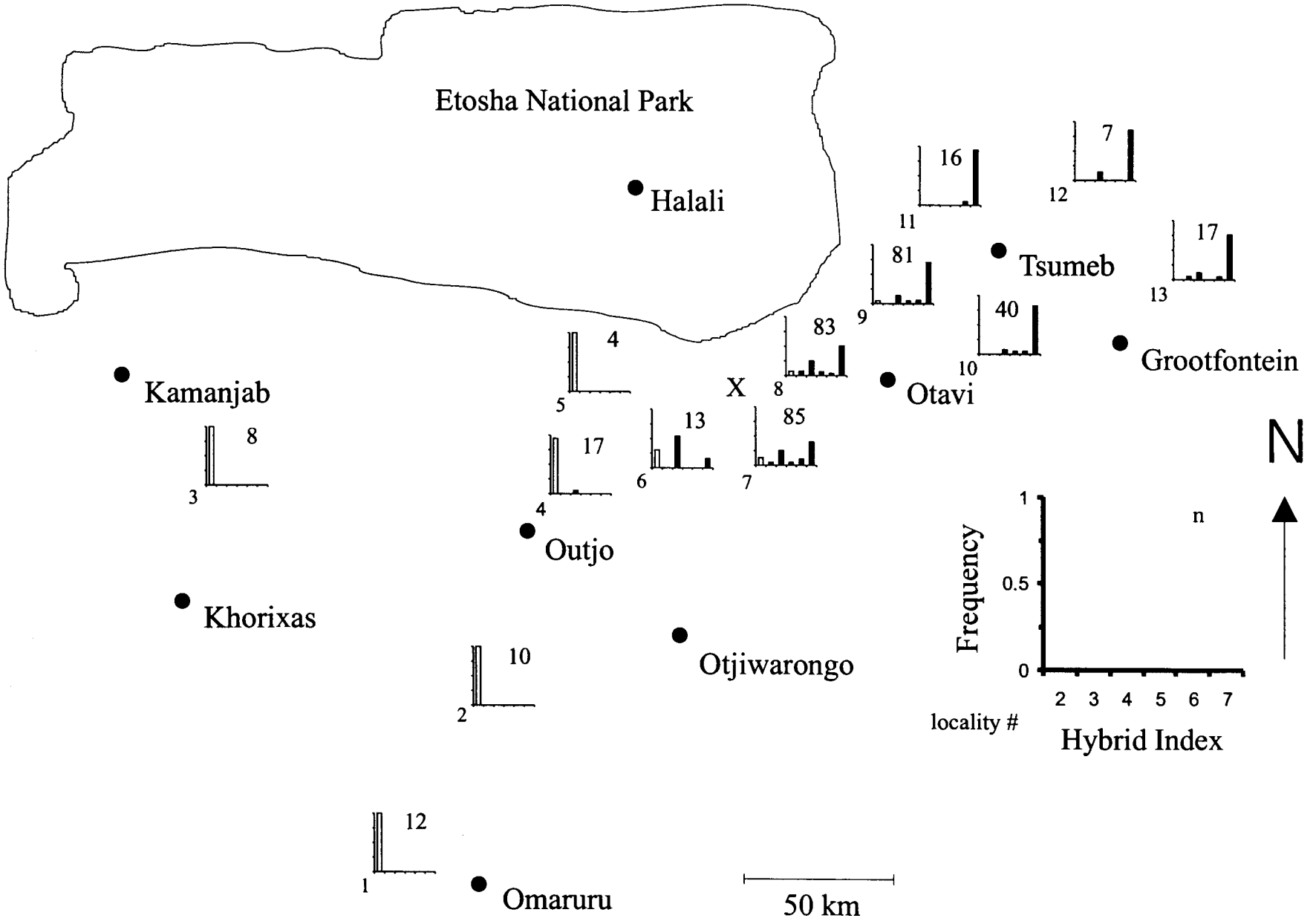



**Figure 3:** Geographic distribution of hybrid index histograms that represent the change in the phenotypic characters, facial plumage and eye colour, across the contact zone. X marks the approximate centre of the contact zone.

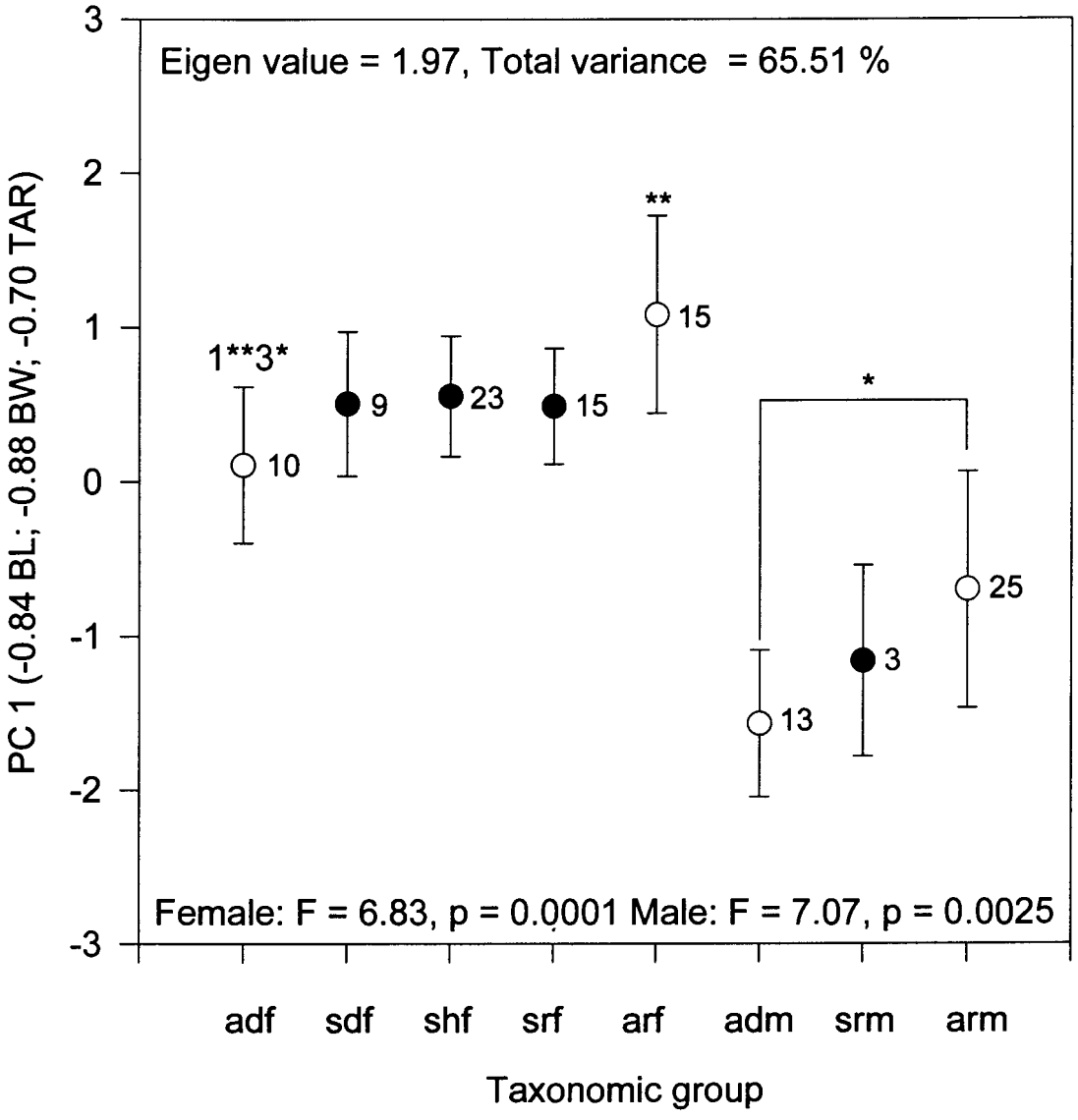



**Figure 4:** Morphometric differences between allopatric (white circles) and sympatric (black circles) representatives of *T. e. rufirostris* and *T. e. damarensis*. Females and males were analysed separately in the *post hoc* comparison of means. Sample sizes are indicated adjacent to the mean for each taxon. Significantly different taxon pairs are shown by lines joining taxa where * = $p < 0.05$ and ** = $p < 0.001$. Where significance indicators occur above the error bars that significance is relevant to the particular taxon compared to all the others of the same sex. PC1 = First principal component with factor loadings of each variable in parenthesis where BL = Bill length, BW = Bill width and TAR = Tarsus length. Taxonomic groups are as follows: df = allopatric *T. e. damarensis* female, sdf = sympatric *T. e. damarensis* female, shf = sympatric hybrid female, srf = sympatric *T. e. rufirostris* female, arf = allopatric *T. e. rufirostris* female, adm = allopatric *T. e. damarensis* male, srm = sympatric *T. e. rufirostris* male and arm = *allopatric T. e. rufirostris* male.

**Table 3:** a) Principal component loadings of the twelve variables in a Principal Component Analysis of calls from allopatric populations of *Tockus erythrorhynchus damarensis* and *Tockus erythrorhynchus rufirostris*. Factor loadings greater than 0.7 are indicated in bold. Cumulative variance is the total cumulative variation accounted for by each principle component.

| Variable | Loading on PC1 | Loading on PC2 | Loading on PC3 |
|---|---|---|---|
| DUR | -0.3206 | **0.8670** | 0.3414 |
| PRO_1 | **0.7219** | 0.6608 | -0.0368 |
| PRO_2 | **-0.7223** | -0.6601 | 0.0385 |
| MDUR_C1 | -0.3186 | -0.1828 | 0.5798 |
| MDUR_BC1 | **0.8933** | -0.1134 | -0.1962 |
| N_C1 | -0.1226 | **0.9682** | 0.1214 |
| N_C2 | **-0.8800** | 0.3945 | 0.0569 |
| C_A | 0.4585 | -0.1111 | 0.4814 |
| B_D | -0.6305 | 0.1462 | 0.0187 |
| C_RATE1 | **-0.8797** | -0.1188 | -0.0086 |
| C_RATE2 | -0.4965 | 0.2621 | **-0.7667** |
| EM_FREQ | 0.0173 | 0.1054 | -0.4907 |
| **Eigen value** | 4.46 | 2.89 | 1.57 |
| **Cumulative Variance (%)** | 37.19 | 61.28 | 74.39 |

b) Summary of the forward Discriminant Function Analysis of twelve call variables from allopatric populations of *Tockus erythrorhynchus damarensis* and *Tockus erythrorhynchus rufirostris*. The discrimination achieved, between allopatric calls of the two subspecies in the DFA, was 100%. F = test statistic, df1 = degrees of freedom in numerator, df2 = degrees of freedom in denominator, p = probability value. Significance at the 95% level is indicated in bold.

| Variable | Step # | Wilk's Lambda | Partial Lambda | F (df1, df2) | P |
|---|---|---|---|---|---|
| B-D | 1 | 0.586 | 0.198 | 43.80 (1, 17) | **<0.001** |
| MDUR_BC1 | 2 | 0.215 | 0.541 | 9.48 (1, 16) | **0.007** |
| EM_FREQ | 3 | 0.176 | 0.661 | 7.69 (1, 15) | **0.014** |

first was to identify typical PC1 values of allopatric *T. e. damarensis* and *T. e. rufirostris* calls (*T. e. damarensis* min = -2.50, max = -0.02, mean = -0.70; *T. e. rufirostris*: min = 0.08, max = 2.19, mean = 1.00). The maximum *T. e. damarensis* and minimum *T. e. rufirostris* PC1 values fortuitously approximate zero for the allopatric populations. Therefore, we assign negative and positive PC1 values as typically *T. e. damarensis* and *T. e. rufirostris* respectively. The second purpose of PCA was to represent change of call structure across the contact zone. The regression analysis of location against PC1 yielded a significant ($F_{1,83}$ = 11.82, $p < 0.001$) positive relationship indicating a change from typically *T. e. damarensis* calls in the southeast to typically *T. e. rufirostris* calls in the northwest. Populations west of Outjo have typical *T. e. damarensis* calls, populations between Outjo and Tsumeb have representatives of both subspecies' calls whilst populations northeast of Tsumeb and Grootfontein have *T. e. rufirostris* type calls (Figure 5). The scatterplot of PC1 values against distance (Figure 6) suggests asymmetrical introgression of vocalisations since PC1 values characteristic *of T. e. damarensis* calls occur further northeast (up to 150 km) than do PC1 values characteristic of *T. e. rufirostris* calls occur southwest (up to 50 km). Furthermore, there are more phenotypic *T. e. rufirostris* birds with typically *T. e. damarensis*, n = 16, calls than *vice versa*, n = 3 (Figure 6).

*Breeding pair composition*

The observed breeding pair combinations indicates that hybridisation in the contact zone is not symmetrical (Table 5). The expected frequencies of each pair combination were calculated from the observed frequencies of individuals (*T. e. rufirostris* = 54%, *T. e. damarensis* = 40%, hybrids = 6%). It is clear that there is an excess of pure *T. e. rufirostris* pairs and pure *T. e. damarensis* pairs, whereas there is a deficiency of hybrid pairs. Furthermore the number of pure *T. e. damarensis* pairs is far greater than expected and the number of female *T. e. rufirostris* – male *T. e. damarensis* pairs is far less than expected. These asymmetries contribute to the very low probability of drawing the observed breeding pair composition from a random mating population (P = 0.000367). Moreover, the binomial probability (k = 34, n = 53, p = 0.5, $P < 0.05$) indicates that there

**Table 4:** Differences between allopatric calls of both subspecies and the calls from the northeastern and southwestern extremes of the contact zone. The matrix represents the results of the Duncan's Multiple Range *post hoc* test of means. The *T. e. damarensis* southwest and *T. e. rufirostris* northeast are calls from the southwestern and northeastern extremes of the contact zone respectively. The allopatric populations of *T. e. damarensis* and *T. e. rufirostris* are calls from Windhoek and South Africa respectively. Sample sizes for each group are indicated in parentheses and significant probability values are indicated in bold.

| | **1.** | **2.** | **3.** | **4.** |
|---|---|---|---|---|
| 1. *T. e. damarensis* southwest (11) | | **0.001** | 0.327 | **<0.001** |
| 2. *T. e. rufirostris* northeast (3) | | | **<0.001** | 0.255 |
| 3. *T. e. damarensis* allopatric (11) | | | | **<0.001** |
| 4. *T. e. rufirostris* allopatric (8) | | | | |

**Table 5:** Composition of breeding pairs from the central 100km of the hybrid zone in northern Namibia. These data were used in the exact test of randomness of mating and in the calculation of hybridisation assymetry between the sexes. O = observed, E = expected number of each pair combination based on the number of individuals of each subspecies*.

| | **Male *T. e. r*** | | **Male hybrid** | | **Male *T. e. d*** | | |
|---|---|---|---|---|---|---|---|
| | **O** | **E** | **O** | **E** | **O** | **E** | |
| **Female *T. e. r*** | 37 | 34.5 | 3 | 3.5 | 16 | 25.5 | 56 |
| **Female hybrid** | 10 | 3.5 | 0 | 0.36 | 0 | 2.6 | 10 |
| **Female *T. e. d*** | 24 | 25.5 | 0 | 2.6 | 27 | 18.9 | 51 |
| **Total pairs** | | | | | | | 117 |

*E = $((M_I/M) \times (F_j/F)) \times (N)$, where E = expected number, $M_i$ = number of males of category i, M = total number of males observed $F_j$ = number of females of category j, F = total number of females observed, N = total number of pairs observed. In each case i and j are assigned to one of the following categories: *T. e. rufirostris*, *T. e. damarensis* or hybrid.

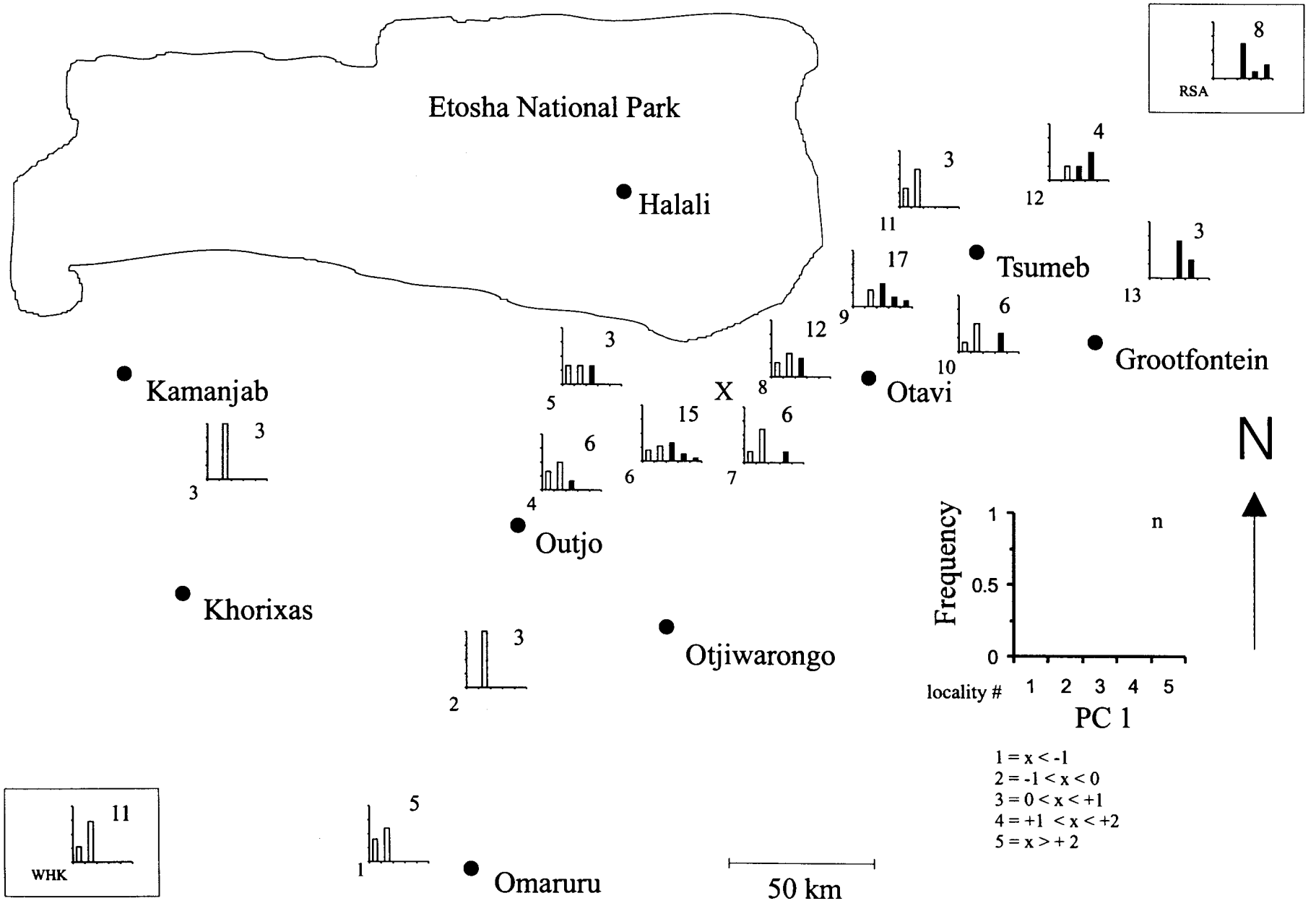



**Figure 5:** Geographic distribution of PC1 histograms that represents the change in call type from phenotypically pure *T. e. damarensis* (southwest) to phenotypically pure *T. e. rufirostris* (northeast). The allopatric populations of both subspecies are also represented as WHK = Daan Viljoen Game Reserve, Windhoek and RSA = South Africa. X marks the approximate centre of the contact zone.

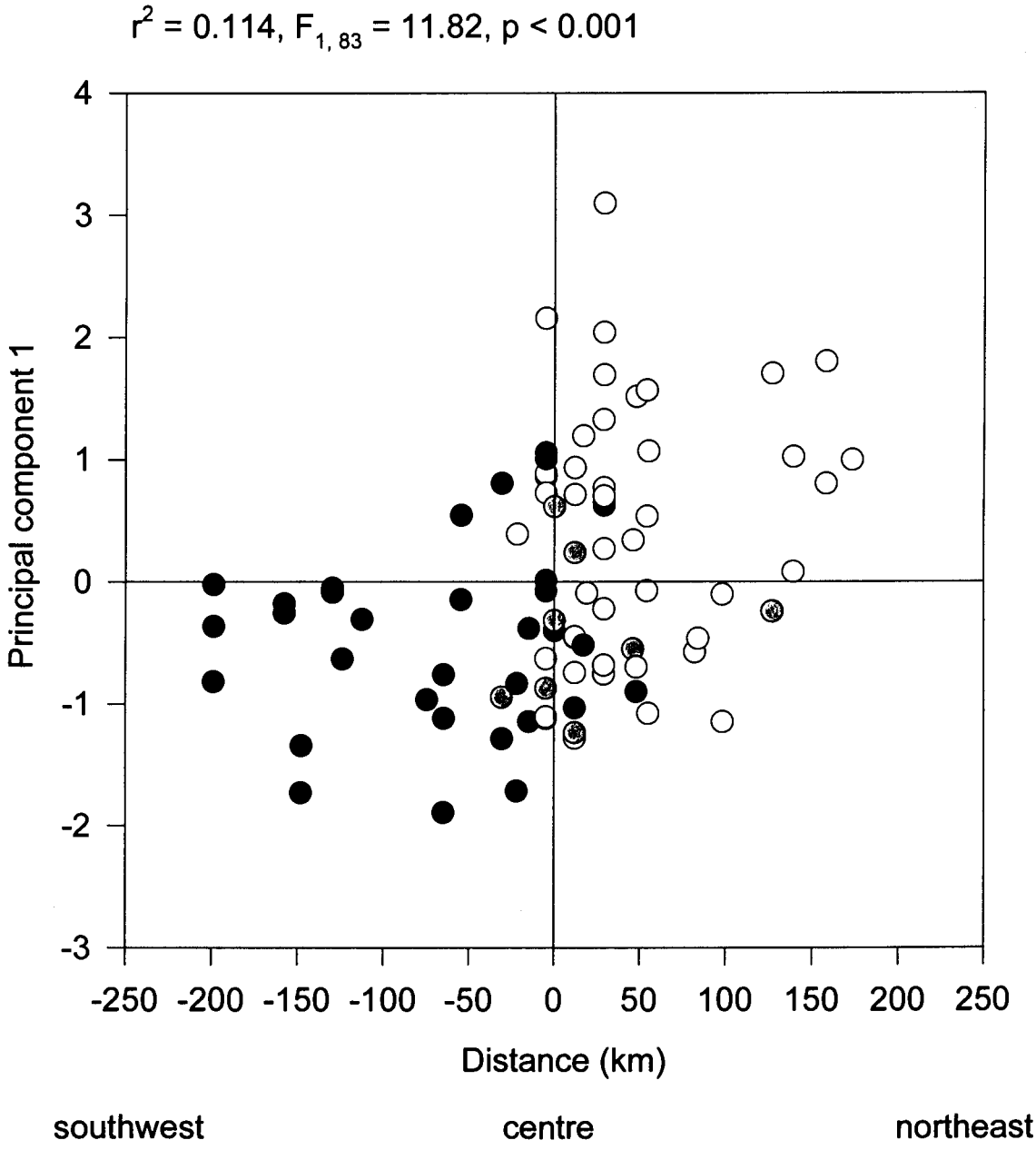



**Figure 6:** Scatterplot of vocalisation PC1 values against distance from the centre of the contact zone. The phenotypes of each of 85 vocalisations are represented. Black circles are *T. e. damarensis* phenotypes, grey circles are hybrid phenotypes and white circles are *T. e. rufirostris* phenotypes.

is a significantly larger proportion of *T. e. rufirostris* males in heterospecific pairs than *T. e. rufirostris* females.

*Breeding biology*

Clutch size and brood size were not significantly different between homo- and heterospecific breeding pairs (Table 6). However the hatching success was significantly lower in heterospecific pairs than in homospecific pairs. None of the fitness attributes of females appeared responsible for the significant differences, since hatching success still yielded a significant difference between homo- and heterospecific pairs even when we included the fitness attributes as covariates (Table 6). However one of the fitness covariates, bill length, did contribute to the significant difference between the brood success of hetero- and homospecific pairs (Table 6). Of the eleven clutches with unhatched eggs, two were from homospecific pairs and nine were from heterospecific pairs. Furthermore, of the twenty-seven successful clutches, seventeen were from homospecific pairs whilst ten were from heterospecific pairs. All the eggs of unsuccessful clutches showed no signs of development.

**Table 6:** Results of the ANOVA and ANCOVA on compatability measures of hybridisation. Fitness covariates include female mass after laying, bill length, bill width, tarsus and egg volume. X homo- = mean of homospecific breeding pairs, X hetero- = mean of heterospecific breeding pairs, F = test statistic, $df_1$ = degrees of freedom in numerator, $df_2$ = degrees of freedom in denominator, P = probability value where significance is indicated in bold.

| | X homo- | X hetero- | F | $df_1$ | $df_2$ | P |
|---|---|---|---|---|---|---|
| **ANOVA** | | | | | | |
| Clutch size | 4.73 | 4.73 | 0.0 | 1 | 36 | P > 0.05 |
| Brood size | 4.63 | 4.32 | 1.36 | 1 | 36 | P > 0.05 |
| Hatching success | 0.98 | 0.90 | 7.03 | 1 | 36 | **P < 0.05** |
| **ANCOVA** | | | | | | |
| Hatching success | 0.98 | 0.90 | 4.33 | 1 | 36 | **P < 0.05** |

| **Fitness attribute** | **beta** | **T** | **P** |
|---|---|---|---|
| Female mass after laying | 0.24 | 1.45 | P > 0.05 |
| Bill length | 0.53 | 2.89 | **P < 0.01** |
| Bill width | -0.07 | -0.44 | P > 0.05 |
| Tarsus length | 0.02 | 0.10 | P > 0.05 |
| Mean egg volume | 0.23 | 1.25 | P > 0.05 |

## Discussion

*Morphological variation*

Allopatric populations of the two subspecies of Redbilled Hornbill considered have consistent diagnosable differences. These morphological differences are eye colour, facial plumage colour and body size. *Tockus e. damarensis* individuals are considerably larger than *T. e. rufirostris* (smaller values on PC1 represent larger bill length, bill width and tarsus length). The morphological characteristics represent a typical cline across the contact zone from southwest to northeast, with intermediary phenotypes occurring in the centre. Barton and Hewitt (1985) have suggested that two processes contribute to the dynamics of hybrid zones: dispersal and selection. In our opinion dispersal both into and out of the contact zone will affect the dynamics of a hybrid zone. Furthermore, Harrison and Rand (1989) have stressed the importance of vegetation in altering the dynamics of a Mosaic Hybrid zone. Both these factors (dispersal and habitat distribution) may influence the Redbilled Hornbill hybrid zone in northern Namibia. Although the two subspecies under consideration do not show obvious alternate habitat preferences as required in the Mosaic model, vegetation still influences the dynamics of this hybrid zone. Hornbills nest in tree cavities (Kemp 1995) and the availability of tree cavities is clearly dependent on vegetation type. The habitat northeast of the contact zone comprises forest savanna and woodland whereas the southwest is largely thornbush and mopane savanna (Figure 7). As a result the abundance of hornbills is greater in the northeast than the southwest. This would allow for proportionally more *T. e. rufirostris* than *T. e. damarensis* individuals to enter the hybrid zone. The dispersal from the hybrid zone may too be asymmetrical and thus account for the observation of hybrid phenotypes further northeast from the centre than southwest.

*Call Variation*

The calls of allopatric *T. e. damarensis* and allopatric *T. e. rufirostris* individuals are consistently distinct. Moreover, the distribution of call types across the contact zone

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

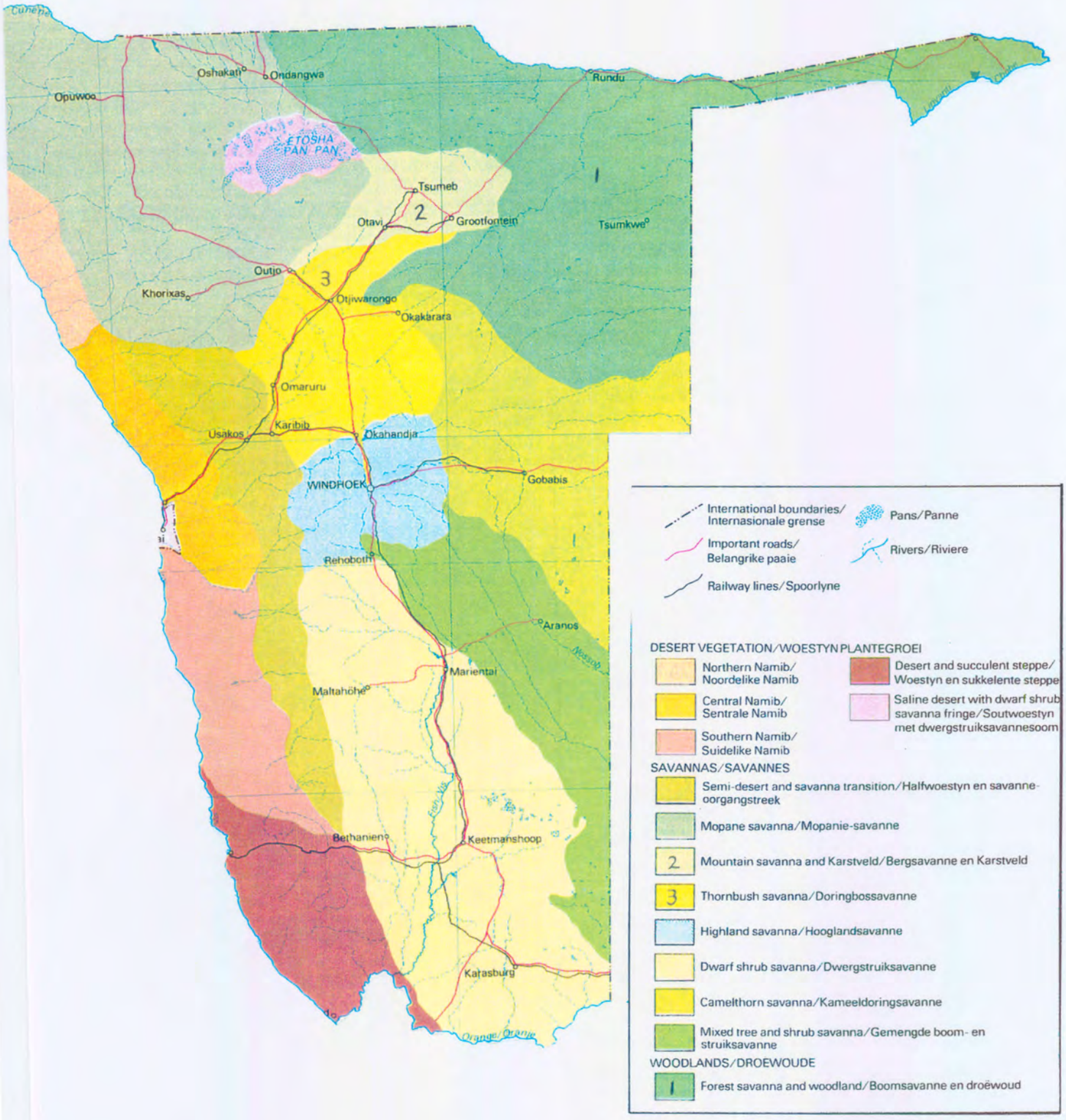



**Figure 7**: Natural vegetation of Namibia (modified from van der Merwe 1983)

mirrors the phenotypic pattern of a change from typical *T. e. damarensis* in the southwest to typical *T. e. rufirostris* in the northeast. In many nonpasserine birds the calls of hybrids are intermediates between the parental species (Payne 1986), attributed to the genetic inheritance of vocalisation in nonpasserines. Delport (2000a Chapter 2) suggested that hybridisation in the contact zone between *T. e. damarensis* and *T. e. rufirostris* is responsible for the unclear discrimination of the calls of these two taxa. However, Delport (2000a Chapter 2) did not distinguish between allopatric and sympatric calls of the two subspecies as we have subsequently done. Typical *T. e. damarensis* calls from allopatry fortuitously have negative principal component values and typical *T. e. rufirostris* calls have positive. The calls in sympatry exhibit the same degrees of variation as those in allopatry (Figure 6). Therefore, on the basis of principal component values, there appear to be no clear intermediate call types in the contact zone. This anomaly may be due to the fact that the variables we have chosen as representative of the taxa do not sufficiently identify intermediate calls. Another possibility is that the genetic determination of call types is limited to a single locus with two alleles where the call of each individual is determined by its combination of alleles. Only one hybrid individual recorded did have a call that was typical of both *T. e. damarensis* and *T. e. rufirostris* (Figure 2). The call of this individual started with single-clucks, proceeded to a phase of typical *T. e. rufirostris* double-clucks and finally ended on a phase of typical *T. e. damarensis* double-clucks. Therefore, hybridisation that results in an intermediate call type appears to occur infrequently.

Call characteristics are distributed asymmetrically in the contact zone: more sympatric *T. e. rufirostris* individuals have *T. e. damarensis* type calls than *vice versa*. May, Endler & McMurtrie (1975) suggest that asymmetric gene flow is responsible for such patterns in hybrid zones. Since the calls of non-passerine birds are presumed to be innate, asymmetric gene flow may account for the observed pattern. However, attributing asymmetric introgression to asymmetric gene flow does not shed light on any of the processes resulting in the observed pattern. Differences in interspecific mating success (Sperling & Spence 1991), mating behaviour (Lamb & Avise 1986), generation time (Barton 1986) and mating preferences (Parsons, Olson & Braun 1993) may account for

asymmetric gene flow. Of these hypotheses both differences in interspecific mating success and mating behaviour would only produce asymmetry in sex-linked traits. We have only measured male calls in this analysis and therefore we can not guarantee that calls are not sex-linked. However, if male calls are sex-linked then an excess of male *T. e. rufirostris* – female *T. e. damarensis* pairs would result in proportionally more males with *T. e. rufirostris* calls than with *T. e. damarensis* calls as observed. This result, coupled with the similar characteristics of male and female calls, suggests that calls are not sex-linked. Another possibility is that the asymmetrical patterns of assortative mating contribute to asymmetrical call inheritance. However, this hypothesis is incomplete without knowledge of the call inheritance mechanisms in Redbilled Hornbills. Finally, sexual selection may account for asymmetric call inheritance where a preference exists for males with a *T. e. damarensis* type of call. Yet this is only an hypothesis that remains to be tested in combination with research into the nature of call inheritance in Redbilled Hornbills.

*Breeding biology and pair composition*

It is thought that mating between species may result in the reduced fitness of hybrids (Mayr 1963, Dobzhansky 1970, Butlin 1989). This notion spurred the concept of Reinforcement (Dobzhansky 1940) which maintains that selection against hybridisation incompatability produces a greater divergence of mate recognition systems in sympatry and consequently, assortative mating. In the Redbilled Hornbill hybrid zone there is evidence for reduced fitness of heterospecific pairs, which may initiate the process of Reinforcement. However, in order for Reinforcement to occur, there needs to be close linkage between loci involved in the mate recognition system and the loci that determine hybrid fitness (Butlin 1989). The inflow of parentals into the hybrid zone would cause greater linkage disequilibrium in the hybrid zone (Butlin 1989) and therefore such an association between loci is unlikely. Furthermore, we are of the opinion that a more parsimonious hypothesis would be that the observed pattern of assortative mating is the result of communication incompatibility between one of two possible crosses between parentals.

## Conclusions

In the latter half of the twentieth century studies of hybrid zones were justified primarily to identify specific status of the taxa involved (Mayr 1963, Rohwer & Wood 1998). The causal factor behind these studies was the prevalence of the Biological Species Concept (Mayr 1963, Dobzhanksy 1970) where species were defined by the absence of interbreeding. However, biologists have since realised that hybridisation is not rare and is particularly widespread among birds (Grant & Grant 1992, Gill 1998). The emphasis of hybrid zone research has since changed to include detailed examinations of the processes occurring within hybrid zones in an attempt to provide a universal hybrid zone model. The Redbilled Hornbill hybrid zone exhibits characteristics of both the Tension Zone Model (Barton & Hewitt 1985) and the Mosaic Model (Harrison & Rand 1989). Barton & Hewitt (1985) suggest that hybrid zones move (i) in the direction of lowest density and (ii) in the preferred direction of dispersal. The stability of the Redbilled Hornbill hybrid zone may be the result of these opposite effects, where the lowest density is in the southwest and the preferred dispersal direction is northeast. Furthermore, we have suggested that habitat determines both the densities of Redbilled Hornbills and their preferred dispersal direction. Therefore, components of the Mosaic (Harrison & Rand 1989) and Tension Zone (Barton & Hewitt 1985) models may be more integrated than previously thought and simply evident at different levels of explanation.

# Chapter 4

Characterisation and evolution of the mitochondrial DNA control region in hornbills (Bucerotiformes)

"Every biological phenomenon is essentially an historical one, one unique situation in the infinite total complex of life."

Delbrück 1966

**Abstract**

We determined the mitochondrial DNA (mtDNA) control region sequences of six Bucerotiformes. Hornbills have the typical avian gene order and their control region is similar to other avian control regions in that it is partitioned into three domains: two variable domains that flank a central conserved domain. Two characteristics of hornbill control region sequence differ from that of other birds. Firstly, domain I is AT rich as opposed to AC rich, and secondly, the control region is approximately 500 bp longer than that of other birds. Both of these deviations from typical avian control region sequence are explainable on the basis of repeat motifs in domain I of the hornbill control region. The repeat motifs probably originated from a duplication of CSB-1 as has been determined in chicken (*Gallus gallus*), quail (*Coturnix japonica*) and snowgoose (*Anser caerulescens*). Furthermore, the hornbill repeat motifs probably arose before the divergence of hornbills from each other, but after the divergence of hornbills from chicken, snowgoose and quail. The mitochondrial control region of hornbills is suitable for both phylogenetic and population studies, with domains I and II probably more suited to population and phylogenetic analyses respectively.

## Introduction

The mtDNA genome has proven to be useful in many avian phylogenetic and population studies (reviewed in Baker & Marshall 1997, Mindell *et al.* 1997, Moore & DeFilippis 1997). Advantages of working with mtDNA include maternal inheritance (Lansman *et al.* 1983); a higher rate of evolution than single copy nuclear DNA (Brown *et al.* 1979) and variable evolutionary rates of genes within the genome itself (Aquadro & Greenburg 1983, Cann *et al.* 1984). Therefore, it is possible to address both phylogenetic and population level questions using different genes of the same genome (Wenink *et al.* 1994). Traditionally, coding genes of the mitochondrial genome, cytochrome b and 12S rRNA, have been reserved for phylogenetic studies (Moore & DeFilippis 1997), whereas the control region has been considered more suitable for population level studies (Baker & Marshall 1997). The control region evolves three to five times faster than the remainder of the mitochondrial genome (Aquadro & Greenburg 1983, Cann *et al.* 1984) and therefore can be difficult to amplify with universal vertebrate primers in a polymerase chain reaction. In addition, both nuclear integrations (Sorenson & Fleischer 1996, Zhang & Hewitt 1996), although not restricted to the control region and repeat motifs (Desjardins & Morais 1990, 1991, Quinn & Wilson 1993, Ramirez *et al.* 1993, Wenink *et al.* 1994, Randi & Lucchini 1998), could render PCR amplification difficult.

Part of the control region is thought to regulate transcription and replication of the vertebrate mtDNA genome (Brown *et al.* 1986, Clayton 1991). These areas that may have function-associated secondary structure should be relatively conserved across species. Such regions that are associated with the termination of replication (Sbisá *et al.* 1997) and the origin of heavy strand replication (CSB-1) have conserved secondary structures in some birds (Quinn & Wilson 1993, Randi & Lucchini 1998). Furthermore, some regions, such as F, D and C-Boxes (Southern *et al.* 1988) and CSB-1, are conserved both within birds and vertebrates. Although the presence of conserved regions should make universal vertebrate primer design simpler, one of these conserved regions (CSB-1) has similar repeat motifs that occur in the 5' domain of Lesser Snowgoose *Anser caerulescens caerulescens*, Domestic Chicken *Gallus gallus* and Japanese Quail *Coturnix japonica* (Quinn & Wilson 1993). In addition to the complications within the avian control region, there are alternate arrangements of

genes surrounding the control region (Quinn & Wilson 1993, Quinn 1997, Mindell *et al.* 1998). Moreover, the alternate arrangements may not be consistent within a genus. Grant (1999) determined that Namaqua Sandgrouse *Pterocles namaqua* have the alternate avian gene order where ND6 is flanked by the control region and a pseudo-control region, as found in the Falconiformes. However, Double-banded Sandgrouse *Pterocles bicinctus* have the general avian gene order, with ND6 flanked on the usptream and downstream sides by cytochrome b and the control region respectively, as found in the Galliformes. Therefore, designing control region primers on the basis of closely related species might be problematic.

A pilot study revealed that published avian control region primers (L16746 - Wenink *et al.* 1994, H400 - Wenink *et al.* 1994, H522 - Quinn & Wilson 1993 & H1254 - Wenink *et al.* 1994) were unsuccessful in amplifying the control region of the hornbills considered in this study. Furthermore, D-Box and CSB-1 primers (L538: 5' CCTCTGGTTCCTATGTCAGG 3' & H875 5' TATGTCCGACAAGCATTCA 3') that were designed in our laboratory on the basis of published chicken, snowgoose and quail sequences (Desjardins & Morais 1990, 1991, Quinn & Wilson 1993) did not amplify the hornbill control region. Therefore, we set out to sequence the bucerotiform control region. Furthermore, in this manuscript we (i) present the complete control region sequence of hornbills, (ii) identify features that hornbills share with other birds and (iii) discuss the origin of features unique to the hornbill control region.

**Methods and Materials**

*Sample collecting*

We obtained samples from seven bucerotiforms; *Tockus leucomelas*, *T. nasutus, T. erythrorhynchus rufirostris*, *T. e. damarensis*, *T. monteiri*, *T. e. kempi* and *Bucorvus leadbeateri*. We collected blood samples of two of the *Tockus* hornbill taxa, *T. leucomelas* and *T. nasutus*, from the Otavi mountain region in northern Namibia. Two of the subspecies of Redbilled Hornbill (*T. e. damarensis* and *T. e. rufirostris*) hybridise in this region (Delport 2000b Chapter 3) and therefore some 200 nestboxes were erected. Although these nestboxes were erected as part of another study (Delport 2000b Chapter 3) they were still useful to obtain some blood samples used in this

study. Furthermore we collected one sympatric male *T. e. rufirostris* from the northeastern extreme of the Namibian contact zone. This sample was the purest *T. e. rufirostris* we could obtain from the contact zone in Namibia, where we had a permit to collect Redbilled Hornbills (Appendix). In addition, one allopatric male *T. e. rufirostris* blood sample was taken from a bird caught in the Northern Province, South Africa. Blood samples of *T. e. damarensis* and *T. monteiri* from the Daan Viljoen Game Reserve, Windhoek (22° 32' S, 16° 58' E) were obtained from C. Boix-Hinzen. Clive R. Barlow supplied one *T. e. kempi* tissue sample collected in The Gambia, West Africa and Alan C. Kemp supplied one *Bucorvus leadbeateri* feather from the Northern Province, South Africa. Blood samples were taken by puncture of the major patagial vein and mixed immediately in blood storage buffer (0.1M Tris-HCl, 0.04M EDTA·$Na_2$, 1.0M NaCl, 0.5% SDS), with a 1:10 ratio of blood to buffer. Muscle, liver and heart tissue samples were frozen at -20° C and feather samples were stored at room temperature.

*DNA extraction*

All blood samples were extracted as follows: 50µl of whole blood was mixed with 450µl extraction buffer (0.05 M Tris-HCl, 0.5 M EDTA·$Na_2$, 1.0M NaCl, 10% SDS). Samples were then digested overnight at 55°C with 0.5mg Proteinase K (Roche Diagnostics); followed by 1 hour at room temperature with 0.1mg RNase A (Roche Diagnostics). Thereafter samples were extracted twice with phenol and once with a chloroform:isoamyl alcohol (24:1) solution. Samples were then precipitated overnight at -20°C in a solution containing 0.1 volumes of 3M Sodium Acetate and 2 volumes of 96% Ethanol. Finally the genomic DNA was pelleted in a tabletop microcentrifuge at 13000rpm and resuspended in 50-100µl Sabax® water. The non-specific amplification of hornbill control region with published avian primers may be the result of nuclear integrations (Zhang and Hewitt 1996, Sorenson & Fleischer 1996). Therefore, we extracted pure mtDNA, with the standard technique of ultracentrifugation through a cesium chloride density gradient (Lansman *et al.* 1981), from heart tissue of the male *T. e. rufirostris* collected in northern Namibia.

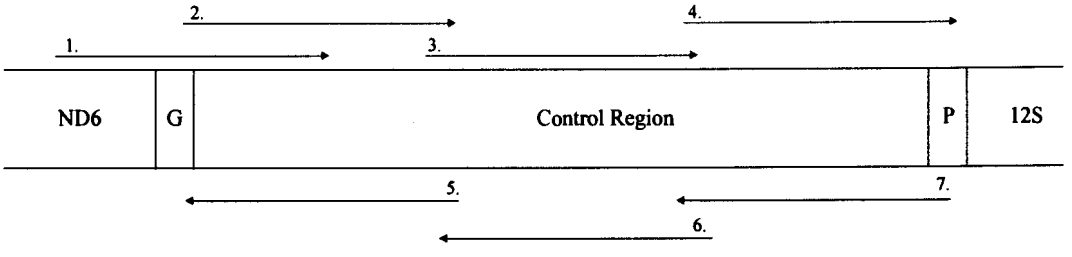
*PCR amplification, cycle sequencing & primer design*

Approximately 50ng of purified mtDNA were used as template in a PCR reaction. Reactions were performed in a total volume of 50 µl in thin-walled 200µl microcentrifuge tubes. The reaction mix contained, in addition to the purified mtDNA, 2mM $MgCl_2$, 5 µl 10 x reaction buffer, 0.2 mM of each of four nucleotides, 12.5 picamol of each primer and 1.5U of Super-therm® DNA polymerase. The primers Thr-L, designed in our laboratory from *T. erythrorhynchus* sequence (Mindell *et al.* 1998, Genbank accession # AF082071), and H1254 (Wenink *et al.* 1994) were used to amplify the ND6 gene and control region of the purified mtDNA. A Geneamp® PCR System 9700 (Applied Biosystems, California) was used to cycle the reaction mix through the following conditions: denaturing at 94°C for 2min; followed by 35 cycles of denaturing at 94°C for 30 sec, primer annealing at 52-58°C for 30 seconds and elongation at 72°C for 90 sec; and finally an extended elongation period of 10 min at 72°C.

Polymerase chain reaction of the pure mtDNA template yielded a single amplified product of approximately 3000 base pairs. Therefore we purified the product with a High Pure™ PCR Product Purification kit (Boehringer Mannheim). The primers Thr-L and H1254 were used when we proceeded with dye-terminator cycle sequencing (Big Dye DNA sequencing kit, PE Applied Biosystems) according to the manufacturers instructions. Sequences of the light and heavy strands, of the pure mtDNA PCR product, were determined up to 600 base pairs from the 5' and 3' ends respectively with an ABI377 automated sequencer (PE Applied Biosystems, California). We designed primers at approximately 500 bp downstream and upstream of the ends of the pure mtDNA PCR product and sequenced with these newly designed primers. We continued this process, of designing primers and sequencing, until the sequences of the heavy and light strands overlapped. Six additional primers were designed in order to sequence through the entire hornbill control region in both directions. These primer names, sequences and annealing positions on the chicken mitochondrial DNA genome are shown in Table 1.

**Table 1**: Primer identities, sequences and characteristics. Universal primer names refer to the position of the 3' base in the chicken mitochondrial genome (Desjardins and Morais 1990). [a]Matches number in Figure. ND6 = NADH dehydrogenase subunit VI, G = transfer RNA-Glu, P = transfer RNA-Phe & 12S = 12S ribosomal RNA. Thr-L is not visually represented, yet it occurs downstream of cytochrome b in tRNA-Thr.

| #[a] | Primer Name | Primer Sequence (5'-3') | Forward/ Reverse | GC:AT | Universal Name |
|---|---|---|---|---|---|
| 1 | HBND6L | CATGAACAGCACCAACGC | Forward | 55:45 | L16412 |
| 2 | HBGluL | TTCTCCAAGGTCTATGGC | Forward | 50:50 | L16743 |
| 3 | HBDL3L | ACACGGATATGCCAGACG | Forward | 55:45 | L350 |
| 4 | HBDL4L | GAGCATACAATGACCGGC | Forward | 55:45 | L742 |
| 5 | HBDL3H | CACCGGGTTGCTGATTTCACG | Reverse | 57:43 | H423 |
| 6 | HBDLH | TGTACGGGAAGTTAGGAG | Reverse | 50:50 | H911 |
| 7 | H1254 | ATCTTGGCATCTTCAGTG | Reverse | 44:56 | H1254 |
| | Thr-L | ACATTGGTCTTGTAAACC | Forward | 39:61 | L16075 |



Genomic DNA extractions were then performed on the remaining blood (allopatric *T. e. rufirostris*, allopatric *T. e. damarensis*, *T. leucomelas* & *T. nasutus*) and tissue (*T. e. kempi* & *B. leadbeateri*) samples. Amplifications of genomic DNA extractions were implemented as before except different primers were used. Polymerase chain reaction of the entire control region with primers HBND6L and H1254 yielded non-specific amplification. Therefore, it was necessary to amplify three separate regions with the following primer combinations: 5' domain - HBND6L & HBDL3H, central domain - HBDL3L & HBDLH and the 3' domain - HBDL4L & H1254 (Table 1). Amplification products were purified, as before, and sequenced in both directions with the respective primers. Amplified products of the 5' domain were sequenced with both HBND6L and HBGluL.

*Sequence analysis*

Sequences were imported into Sequence Navigator (PE Applied Biosystems) and proof read. Consensus sequences of the entire control region for each taxon were thereafter aligned in CLUSTAL W version 1.74 (Higgins *et al.* 1992). Aligned sequences were imported into PAUP version 4.0 (Swofford 1999) where nucleotide frequencies and numbers of transitions and transversions were calculated. The transition-to-transversion ratios expected at saturation were calculated from empirical base frequencies with Holmquist's (1983) formula:

$$R_{s/v} = (p_a \cdot p_g + p_c \cdot p_t)/(p_a + p_g)(p_c + p_t)$$

where $p_i$ is the frequency of base $i$. We used a t-test of independent samples to determine whether the observed within and between species transition-to-transversion ratios were significantly different from that expected at saturation. Phylogenetic analyses were implemented using PAUP (Swofford 1999) and Neighbour-Joining trees (Saitou & Nei 1987) were constructed using the absolute number of differences. Robustness of phylogenetic trees was assessed using the bootstrap (Felsenstein 1985) with 1000 replicates. Putative secondary structures within the control region were determined using the computer program RNAstructure (Mathews *et al.* 1999) with the energy parameters specified by SantaLucia (1998). Thereafter, we used the computer program RNAdraw (Hofacker *et al.* 1995) to visualise secondary structures.

## Results

*Control region characteristics*

The complete control region was sequenced for only six of the eight bucerotiforms samples we obtained (Figure 1). Complete sequences of *T. nasutus* and *T. monteiri* are not presented, since we only managed to sequence two thirds of these control regions as a result of failed PCR reactions. We sequenced both allopatric and sympatric samples of *T. e. rufirostris* and, for simplicity, we will refer to these as independent taxa for the remainder of the manuscript. The length of the control region in hornbills is variable where *T. leucomelas* has the longest sequence (1761bp), followed by *T. e. kempi* (1732bp), *B. leadbeateri* (1731bp), *T. e. rufirostris* (1730bp) and *T. e. damarensis* (1729bp). Furthermore, the control region in hornbills appears to be subdivided into three domains (Figure 1). The first domain at the 5' end (nt25-819) is highly variable with 16% variable sites (including indels) among the bucerotiforms. Domain I is further characterised by an AT rich base composition and a deficiency of light strand G nucleotides (Table 2). The central domain (nt820-1276) with 2.7% variable sites is CT rich (Table 2) and generally conserved across the Bucerotiformes. Finally the 3' end (Domain II: nt1277-1793) with 18.6% variable sites is characterised by high proportions of A and T, yet is very low in light strand G nucleotides (Table 2). Chi-square tests of differences in base composition between the six taxa were non-significant (Control Region: $\chi^2 = 2.21$, df = 15, $P > 0.05$, Domain I: $\chi^2 = 0.93$, df = 15, $P > 0.05$, Central Domain: $\chi^2 = 0.33$, df = 15, $P > 0.05$ and Domain II: $\chi^2 = 3.89$, df = 15, $P > 0.05$).

Transition-to-transversion ratios both within and between species are generally high (Table 3). Furthermore, the transition-to-transversion ratios observed within species were significantly greater than those at which saturation is expected (Table 3). This result was true for the entire control region and for each of the three domains (Table 3). Similarly, transition-to-transversion ratios observed between species were significantly greater than those at which saturation is expected (Table 3). However, this result was not consistent across all domains since the central domain has more transversions than transitions in some of the between species comparisons (Table 3).

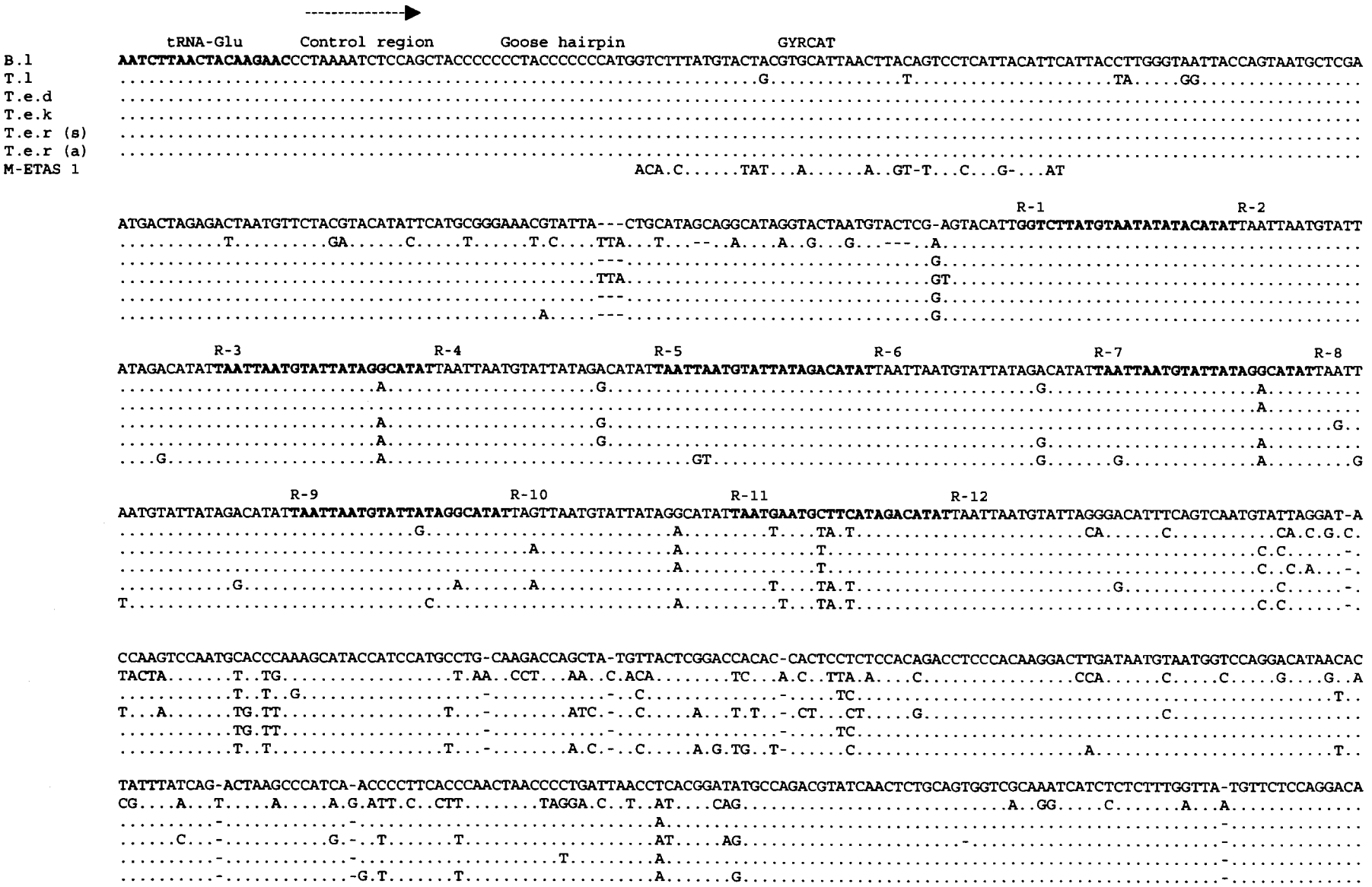
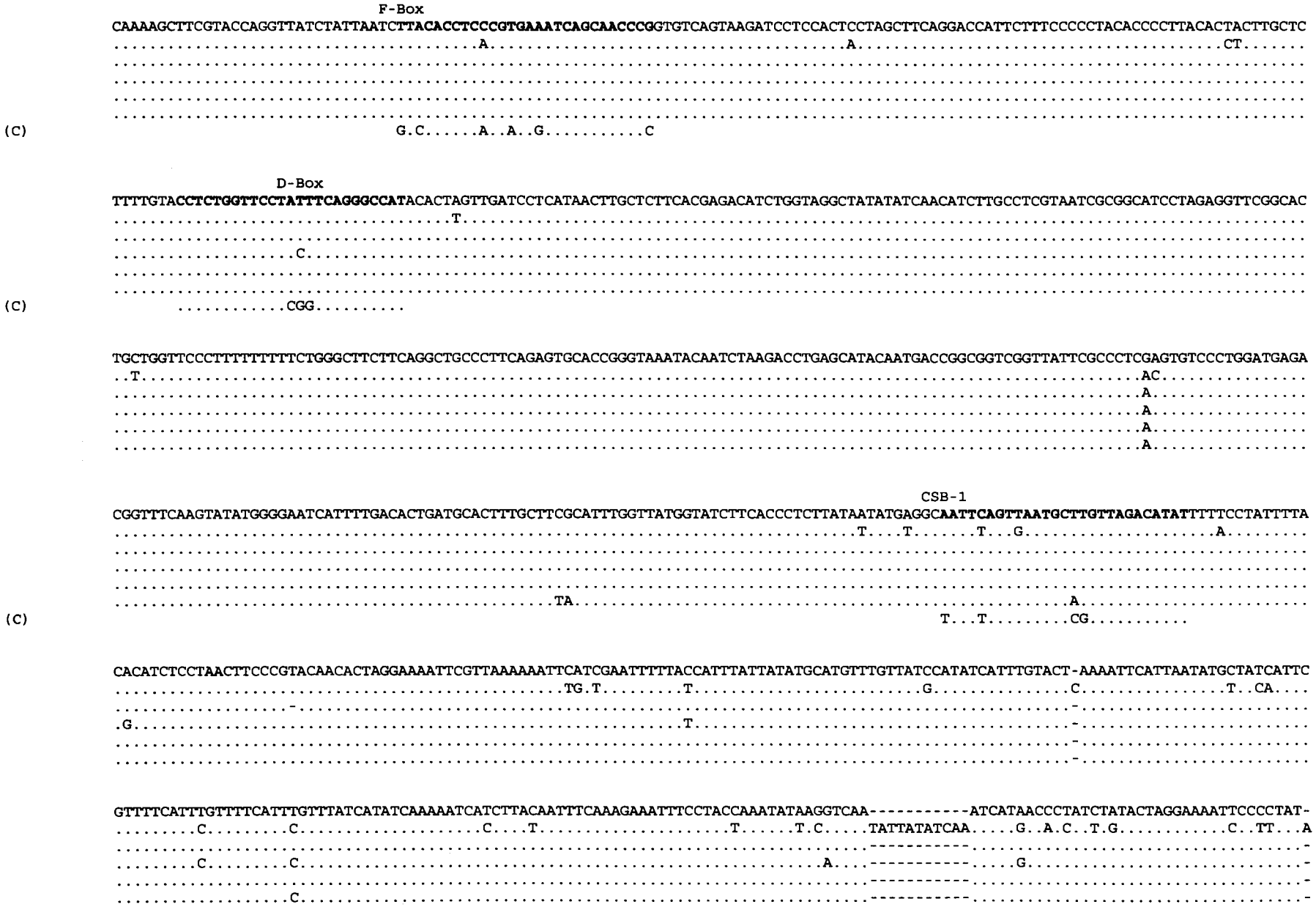



**Figure 1**: Caption on page 63

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA




UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

**Figure 1**: Caption on page 63

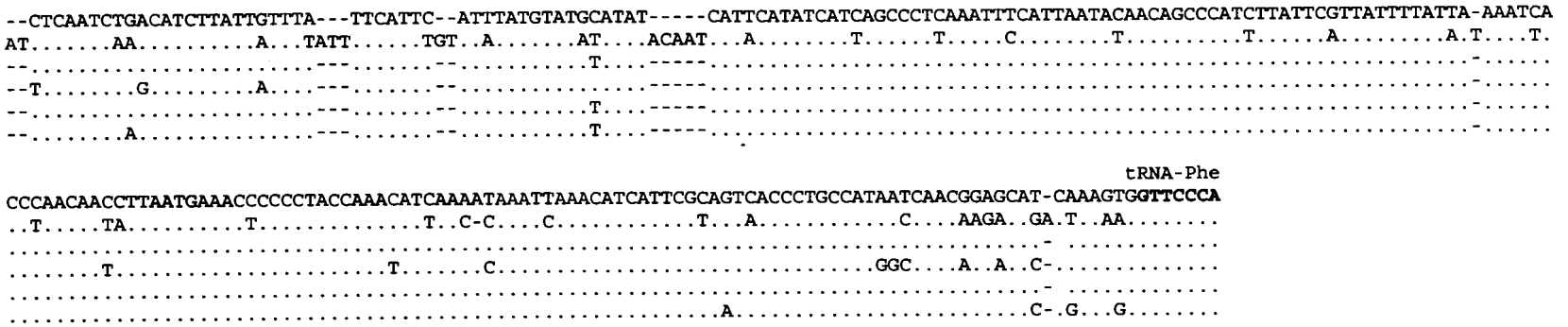



**Figure 1**: Sequence comparison of the control region from six Bucerotidae taxa. B.l = *Bucorvus leadbeateri*, T.l = *Tockus leucomelas*, T.e.d = *Tockus erythrorhynchus damarensis*, T.e.k = *Tockus erythrorhynchus kempi*, T.e.r (s) = *Tockus erythrorhynchus rufirostris* sympatric with *Tockus erythrorhynchus damarensis* and T.e.r (a) = allopatric *Tockus erythrorhynchus rufirostris*. The sequence orientation is from 5' to 3' on the light strand. Dots (.) indicate identity with the *Bucorvus leadbeateri* sequence and dashes (-) indicate gaps. Adjacent tRNA's are represented in bold at the start and end of the sequence, as are the conserved F-box, D-box and CSB-1. Chicken (C) sequences (Desjardins & Morais 1990) corresponding to the conserved F-box, D-box and CSB-1 are shown below the respective regions. The putative goose hairpin (Quinn & Wilson 1993) at the 5' end of the control region is indicated as is the GYRCAT motif that forms part of the extended termination associated sequence (Sbisà et al 1997). Consensus mammalian ETAS-1 sequence (M- ETAS 1), shown below the respective regions, is from Foran et al (1988). Numbers in the right hand margin indicate absolute sequence length. The Bucerotidae control region is partitioned into three domains where nucleotide numbers (nt) refer to the absolute numbers in the right hand margin. Domain I (nt25-919) is highly variable between the six Bucerotidae taxa and contains the 23bp repeat motifs. The central domain (nt920-1360) is conserved across the six Bucerotidae taxa and domain II (nt1361-1893) is more variable than both domain I and the central domain.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

**Table 2**: Base composition of the control region, and respective domains, of six Bucerotiforme taxa.

| | Nucleotide Frequency | | | |
|---|---|---|---|---|
| | A | C | G | T |
| **Control region** | | | | |
| *Bucorvus leadbeateri* | 30.3 | 22.9 | 13.6 | 33.1 |
| *Tockus leucomelas* | 31.4 | 21.3 | 13.2 | 34.0 |
| *Tockus erythrorhynchus damarensis* | 30.6 | 22.8 | 13.5 | 33.1 |
| *T. e. kempi* | 30.4 | 22.6 | 13.7 | 33.4 |
| *T. e. rufirostris* (sympatry) | 30.4 | 22.5 | 13.6 | 33.4 |
| *T. e. rufirostris* (allopatry) | 30.5 | 22.7 | 13.7 | 33.1 |
| Mean | 30.6 | 22.5 | 13.6 | 33.4 |
| Standard deviation | 0.405 | 0.589 | 0.187 | 0.350 |
| **Domain I** | | | | |
| *Bucorvus leadbeateri* | 32.5 | 22.1 | 13.9 | 31.4 |
| *Tockus leucomelas* | 33.7 | 20.9 | 13.7 | 31.6 |
| *Tockus erythrorhynchus damarensis* | 32.8 | 22.0 | 13.8 | 31.4 |
| *T. e. kempi* | 32.7 | 21.2 | 14.0 | 32.1 |
| *T. e. rufirostris* (sympatry) | 32.6 | 21.5 | 14.0 | 31.8 |
| *T. e. rufirostris* (allopatry) | 32.4 | 21.6 | 14.4 | 31.6 |
| Mean | 32.8 | 21.6 | 14.0 | 31.7 |
| Standard deviation | 0.471 | 0.459 | 0.242 | 0.266 |
| **Central domain** | | | | |
| *Bucorvus leadbeateri* | 21.8 | 25.6 | 19.0 | 33.6 |
| *Tockus leucomelas* | 21.1 | 25.6 | 19.0 | 34.2 |
| *Tockus erythrorhynchus damarensis* | 22.0 | 25.7 | 18.9 | 33.4 |
| *T. e. kempi* | 21.8 | 25.9 | 19.0 | 33.3 |
| *T. e. rufirostris* (sympatry) | 22.0 | 25.6 | 18.8 | 33.6 |
| *T. e. rufirostris* (allopatry) | 22.4 | 25.4 | 18.6 | 33.6 |
| Mean | 21.9 | 25.6 | 18.9 | 33.6 |
| Standard deviation | 0.428 | 0.163 | 0.160 | 0.312 |
| **Domain II** | | | | |
| *Bucorvus leadbeateri* | 34.7 | 21.8 | 7.6 | 35.9 |
| *Tockus leucomelas* | 37.1 | 18.2 | 6.7 | 38.0 |
| *Tockus erythrorhynchus damarensis* | 34.7 | 21.6 | 7.6 | 36.1 |
| *T. e. kempi* | 34.7 | 22.1 | 7.6 | 35.6 |
| *T. e. rufirostris* (sympatry) | 34.7 | 21.6 | 7.6 | 36.1 |
| *T. e. rufirostris* (allopatry) | 34.9 | 22.1 | 7.6 | 35.4 |
| Mean | 35.1 | 21.2 | 7.5 | 36.2 |
| Standard deviation | 0.967 | 1.503 | 0.367 | 0.933 |

**Table 3:** T-test of expected transition-to-transversion ratios at saturation versus the observed transition-to-transversion ratios within and between bucerotiform species. $R_{s/v}$ = transition-to-transversion ratio expected at saturation, $W_{s/v}$ = observed transition-to-transversion ratio within species, $B_{s/v}$ = observed transition-to-transversion ratios between species, × = arithmetic mean, t = test statistic, df = degrees of freedom, p = probability level, ns = not significant. Sample sizes are not equal in all instances since some comparisons between taxa yielded no transversions (especially in the central control region) and therefore it was impossible to calculate transition-to-transversion ratios for those taxon pairs.

| Expected | Observed | Expected × | Observed × | t | df | p |
|---|---|---|---|---|---|---|
| **Entire control region** | | | | | | |
| $R_{s/v}$ | $W_{s/v}$ | 0.47 | 6.75 | 4.13 | 10 | <0.05 |
| $R_{s/v}$ | $B_{s/v}$ | 0.47 | 5.04 | 2.32 | 13 | <0.05 |
| **Domain I** | | | | | | |
| $R_{s/v}$ | $W_{s/v}$ | 0.46 | 5.14 | 5.24 | 10 | <0.001 |
| $R_{s/v}$ | $B_{s/v}$ | 0.46 | 4.26 | 2.48 | 13 | <0.05 |
| **Central domain** | | | | | | |
| $R_{s/v}$ | $W_{s/v}$ | 0.53 | 2.67 | 4.90 | 7 | <0.05 |
| $R_{s/v}$ | $B_{s/v}$ | 0.53 | 0.96 | 1.06 | 10 | ns |
| **Domain II** | | | | | | |
| $R_{s/v}$ | $W_{s/v}$ | 0.42 | 9.67 | 3.02 | 7 | <0.05 |
| $R_{s/v}$ | $B_{s/v}$ | 0.42 | 3.03 | 4.37 | 10 | <0.05 |

*Conserved motifs of the avian control region*

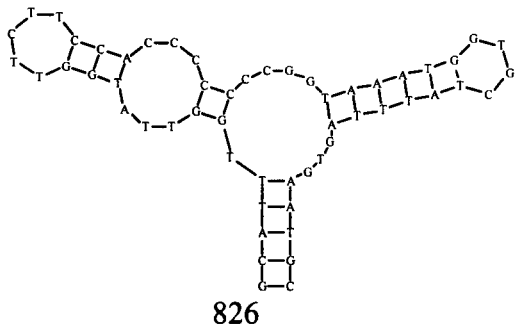
A comparative analysis of alignment (Figure 1) enables the identification of conserved motifs and putative secondary structures, within the bucerotiform control region. The putative goose hairpin of hornbills has a loop structure (Figure 2) yet lacks the hairpin characteristic of chicken and snow goose (Figure 2, Quinn & Wilson 1993). Both the ETAS-1 and GYRCAT motifs (Sbisá *et al.* 1997) are, however, present in the hornbill control region (Figure 1). In addition to these conserved avian motifs the 5' end of the hornbill control region contains a series of 23bp repeats that have secondary structure (Figure 2). The central conserved area of the hornbill control region contains the F-Box and D-Box as found in other avian control regions (Desjardins & Morais 1990, 1991, Quinn & Wilson 1993). Finally the CSB-1 box occurs in the most 5' end of Domain II and has an associated cruciform secondary structure as in the chicken (Figure 2).

*Repeat motifs in Domain I*

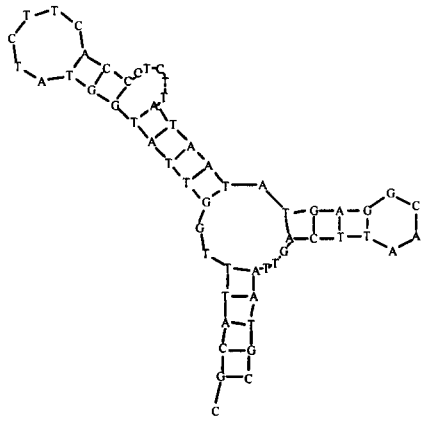
The 5' end of the control region contains a series of 23bp tandem repeats in all eight of the bucerotiform taxa we sequenced. Both the size of each repeat motif (23bp) and the number of motifs (twelve) are conserved across the eight bucerotiform taxa (Figure 3, Table 4). Furthermore, there are 16 different types of repeat motif, which differ from one another by one to seven point mutations (Figure 3). The orders in which these repeat types occur are similar between the taxa (Table 4). Furthermore, it appears that the downstream repeat motifs are more variable between species than those upstream (Table 4). Alignment of the sixteen types of motifs shows similarity with hornbill, chicken, snowgoose and quail CSB-1. In addition the hornbill motifs are similar to those found in the 5' end of chicken (Desjardins & Morais 1990), snowgoose (Quinn & Wilson 1993) and quail (Desjardins & Morais 1991) control regions. Neighbour-joining analysis of the hornbill motifs included with hornbill, snowgoose and chicken CSB-1 indicated that hornbill CSB-1 is basal to a single group of hornbill motifs (Figure 4).

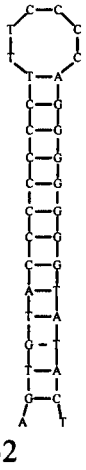
UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

*Gallus gallus* | *Bucorvus leadbeateri*

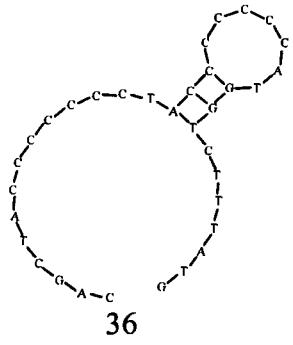


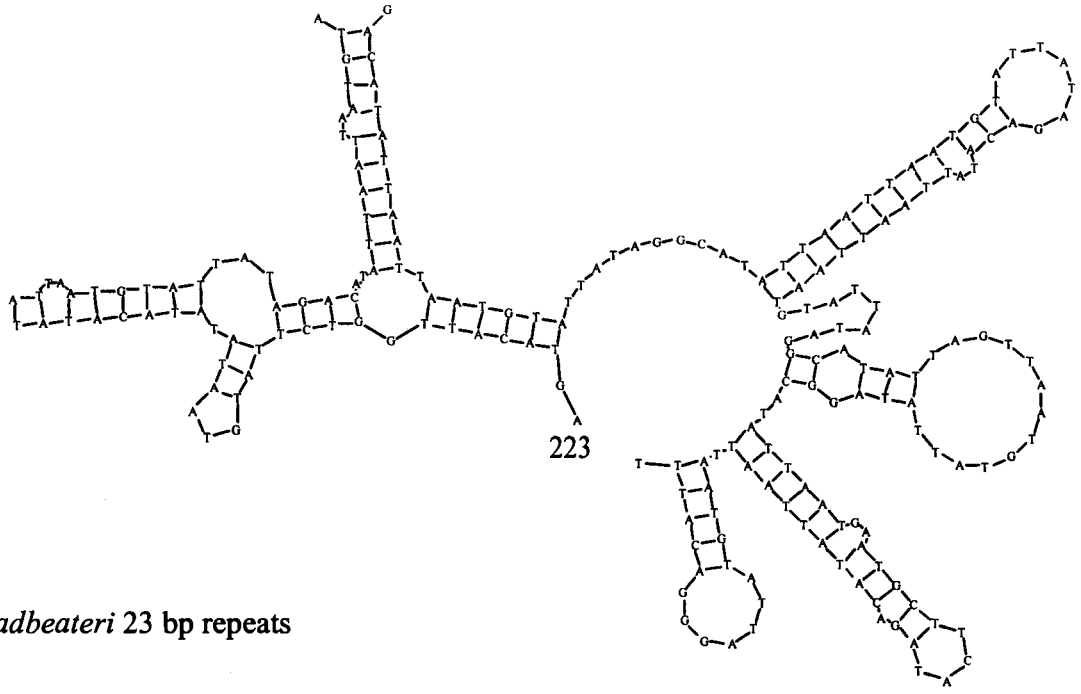

CSB-1




Putative Goose Hairpin




*Bucorvus leadbeateri* 23 bp repeats

**Figure 2:** Secondary structures within the control regions of *Gallus gallus* and *Bucorvus leadbeateri*. Nucleotide positions of *Gallus gallus* are as in Desjardins & Morais (1990) whereas *Bucorvus leadbeateri* positions are as per Figure 1.

```
repeat A        -------TAATTAATGTATTATAGACATAT
repeat B        -------.................G.....
repeat C        --------..G..................
repeat D        --------....GT..............
repeat E        -------.....................C
repeat F        -------............G...G.....
repeat G        -------.............C..G.....
repeat H        --------..G............G.....
repeat I        --------....G.....T.C........
repeat J        --------....G....CT.C........
repeat K        -------.............C......TC
repeat L        -------.............GG.....T.
repeat M        -------.............GG.G...T.
repeat N        --------..G...........C.......C
repeat O        -------GGTC.T.....A....T......
repeat P        -------AGTC.T.....A....T......
T.e.r CSB-1     ---AATTC.G......CT.GT.........
B.l CSB-1       ---AATTC.G......CT.GT.........
G.g CSB-1       ---TATT..G.G....CT.GTCG.......
C.j CSB-1       ---TATA..G.G....CT.GCCG.......
A.c.c_CSB-1     ---TATTGG..G....CTCGT.G......-
chicken_1       -GTCCAT.CTA.GCATG..CTAG......-
quail_1         --GTACA.T.G.GCATGC.CCA.......-
goose_1         CACTACAAC..G....CTC..GGAC....-
chicken_2       CACCTAACT..G....GT.ACAG......-
quail_2         ACCATAACT..G....GT.GCAG......-
goose_2         CCCATAT....G....CT.ACAG......-
```

**Figure 3:** Alignment of Hornbill repeat motifs A through P with (i) CSB-1 of *Tockus erythrorhynchus rufirostris* (*T.e.r* CSB-1), *Bucorvus leadbeateri* (*B.l* CSB-1), *Gallus gallus* (*G.g* CSB-1), *Coturnix japonica* (*C.j* CSB-1), *Anser caerulescens caerulescens* (*A.c.c* CSB-1) and (ii) with repeat motifs from chicken (*Gallus gallus*), Japanese Quail (*Coturnix japonica*) and lesser snow goose (*Anser caerulescens caerulescens*). Dots (.) indicate identity with Repeat A and dashes (-) indicate gaps. Sequences of unequal length were supplemented with gaps, at either the beginning or end, in the phylogenetic analysis. Sequence orientation is 5' to 3' of the light-strand.

**Table 4:** Light-strand (5'-3') order of the sixteen repeat motifs in the eight Bucerotiformes sequenced. Repeat numbers are as in Figure 1 and repeat types are as in Figure 4.

| **Taxon** | **Repeat Number and Type** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| *B. leadbeateri* | O | A | B | A | A | A | B | A | B | H | J | L |
| *T. leucomelas* | O | A | A | B | A | B | A | A | F | C | A | K |
| *T. e. damarensis* | O | A | B | A | A | A | A | A | B | A | I | L |
| *T. e. kempi* | O | A | A | B | A | A | B | C | B | C | I | L |
| *T. e. rufirostris* – sympatry | O | A | A | B | A | B | A | B | A | B | A | M |
| *T. e. rufirostris* – allopatry | O | B | A | A | D | B | C | D | G | C | D | L |
| *T. monteiri* | P | A | A | B | C | A | E | C | F | C | A | L |
| *T. nasutus* | P | A | A | A | A | C | A | F | C | A | F | N |

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

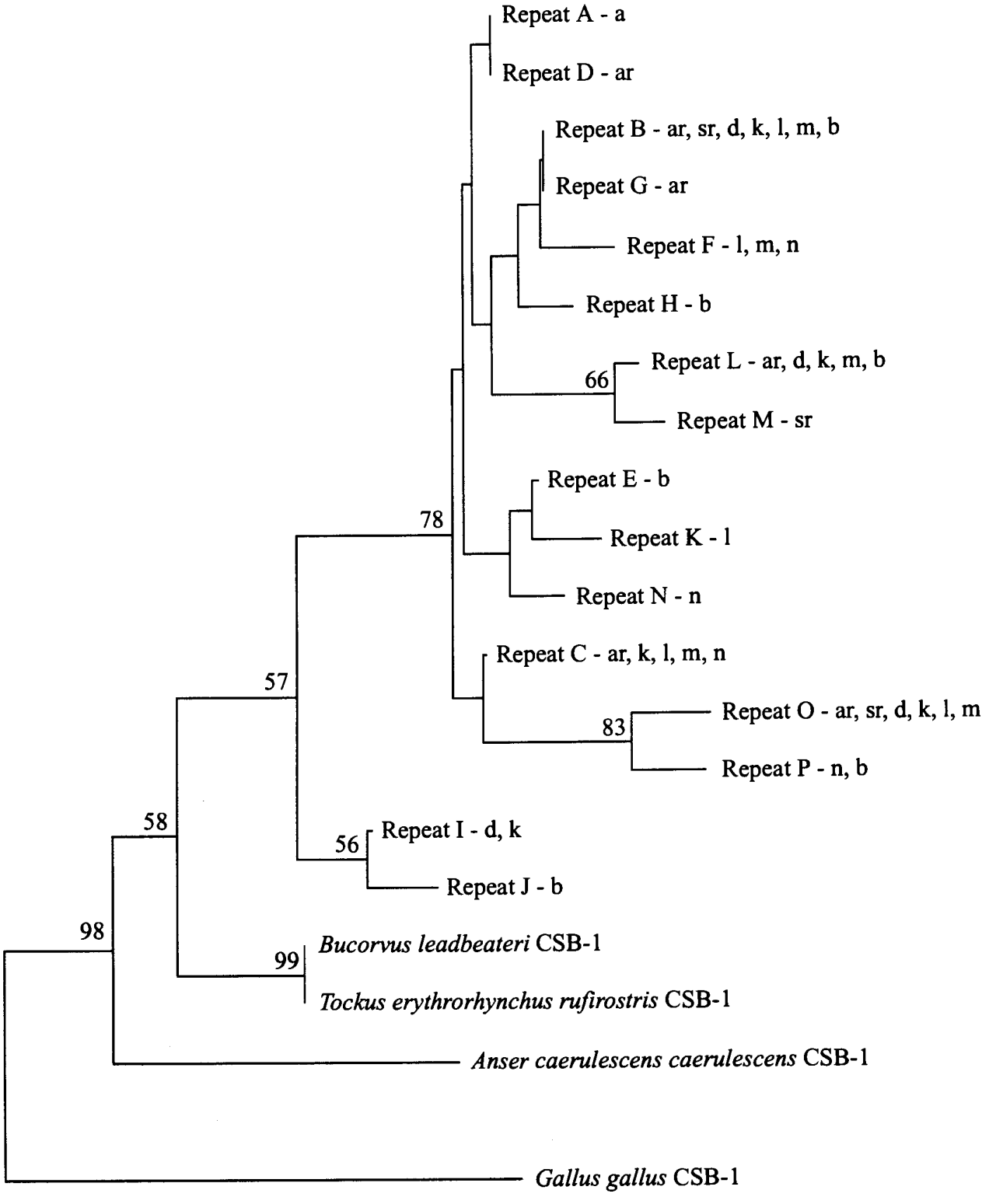



**Figure 4:** Neighbour-joining tree of hornbill repeat motifs and CSB-1. The tree was constructed according to Saitou & Nei (1987) using the absolute number of differences, between the sequences in Figure 3,with gaps included as fifth state characters. One thousand bootstrap replicates were performed and values greater than 50 are indicated at respective nodes. A neighbour-joining tree which included repeat motifs found in Japanese quail (Desjardins & Morais 1991), domestic chicken (Desjardins & Morais 1990) and lesser snowgoose (Quinn & Wilson 1993) was also constructed using the same method. This second tree was essentially the same except there was not sufficient bootstrap support of the hornbill repeat motifs monophyletic group (see Discussion). The taxa in which each repeat motif occurs are indicated. a = all, ar = allopatric *T. e. rufirostris*, sr = sympatric *T. e. rufirostris*, d = *T. e. damarensis*, k = *T. e. kempi*, l = *T. leucomelas*, m = *T. monterei*, n = *T. nasutus* and b = *Bucorvus leadbeateri*.

## Discussion

*Control region characteristics and evolution*

The hornbill control region is similar to other avian control regions in that it comprises three domains that have variable rates of change (Baker & Marshall 1997). Each of these three domains has the following general avian characteristics. Domain I is AC rich and highly variable, the central domain which is responsible for the three-strand displacement loop evident in replication is CT rich and well conserved and finally the variable Domain II is AT rich and low in light strand G (Baker & Marshall 1997). In general, the domains of the hornbill control region are similar to these characteristics. However, hornbill control region is different from other avian control regions in three respects. Firstly, Domain I is AT rich in hornbills as opposed to AC rich in other birds (Baker & Marshall 1997). Secondly, the hornbill control region differs from other avian control regions in size. Hornbill control regions are approximately 1760 bp long whereas that of other birds varies between 1000 and 1400bp (Baker & Marshall 1997, Randi & Lucchini 1998, Saunders & Edwards 2000). Both these deviations of hornbill control region from general avian control region features are probably the result of repeats of CSB-1 in the 5' end of the control region. The 12 repeats of 23bp each increase the length of the hornbill control region by 276 bp. Furthermore, the repeat motifs comprise approximately 40% T nucleotides (Figure 3) and thus also alter base composition. Several authors have found similar repeats in Domestic Chicken (Desjardins & Morais 1990), Japanese Quail (Desjardins & Morais 1991), Lesser Snowgoose (Quinn & Wilson 1993), Peking Duck (Ramirez *et al.* 1993) and *Alectoris* Partridges (Randi & Lucchini 1998). The repeats found in these birds occur at most three times. Therefore, the third difference between hornbill and general avian control region features is that hornbill repeat motifs are consecutive and occur more frequently.

Quinn & Wilson (1993) suggested that a duplication of CSB-1 and migration to the 5' end occurred in chicken, quail and snowgoose before the divergence of these groups from one another. The CSB-1 motifs were then again duplicated after the divergence of the aforementioned taxa from one another (Quinn & Wilson 1993). These independent events would therefore account firstly, for ancestral repeat types common to snowgoose, chicken and quail and secondly, for derived repeat types unique to the

chicken. The phylogenetic analysis of hornbill repeat motifs and CSB-1, from chicken, snowgoose and hornbills, indicate that hornbill CSB-1 is paraphyletic with a single group of hornbill repeat motifs (Figure 4). The Neighbour-Joining analysis that included snowgoose, chicken and quail repeats gave essentially the same tree topology, with hornbill CSB-1 paraphyletic with a single group of hornbill repeat motifs. However, in this analysis, the relationship between hornbill CSB-1 and the hornbill repeat motifs was not supported with bootstrap analysis. This low bootstrap support is not surprising since the repeats in all taxa have probably arisen from a duplication of CSB-1, which is conserved across birds. Although both analysis had low bootstrap support at some nodes, it is certain that the hornbill repeat motifs are more closely related to hornbill CSB-1 than to that of chicken or snowgoose. Therefore, these motifs have arisen after the divergence of the Bucerotiformes from the aforementioned taxa.

*Putative secondary structures*

The vertebrate control region is thought to regulate replication and transcription of the mitochondrial genome (Brown *et al.* 1986, Clayton 1991, Saccone *et al.* 1991). Several authors have identified conserved regions with associated secondary structure within avian control regions (Quinn & Wilson 1993, Marshall & Baker 1997, Randi & Lucchin 1998). Similarly, the hornbill control region contains conserved areas that have secondary structure which may also be functionally important. Firstly, the 5' end of the control region may be involved in the termination of replication (Sbisá *et al.* 1997). Sbisá *et al.* (1997) identified two long conserved sequence blocks (ETAS-1 and ETAS-2) formerly described as TAS elements (Foran *et al.* 1988), in an extensive survey of mammalian control regions. Alignment with a mammalian consensus ETAS-1 sequence (Sbisá *et al.* 1997) indicates that some of the sequence is conserved between mammals and hornbills and therefore may be important in terminating replication of the hornbill control region. Secondly, the goose hairpin (Quinn & Wilson 1993) which also occurs in the 5' region is present in hornbills yet the structure is more of a loop than a hairpin. A series of consecutive C's and G's defines the hairpin structure in chicken and goose (Quinn & Wilson 1993). However several of the G's have been substituted with C's in hornbills, and thus the hairpin has been reduced to a loop. Finally, the sequence and cruciform structure associated with CSB-1 may be involved in the origin of heavy strand replication (Brown *et al.* 1986). The

hornbill CSB-1 region and the CSB-1 type repeats have cruciform secondary structure. The repeats may be important functionally, a notion that is supported by conservation of the number of repeats across eight hornbill taxa. However the repeat sequences themselves are variable between species and the conserved number of repeats may be an artifact of a recent origin of repeats in hornbills.

*Population and phylogenetic utility of the control region*

Several authors have addressed both phylogenetic (Zink *et al.* 1998, Freeland & Boag 1999, Kimball *et al.* 1999, Sato *et al.* 1999) and population level (Lucchini & Randi 1998, Questiau *et al.* 1998, 1999, Marshall & Baker 1999, Piertney *et al.* 2000) questions using avian control region sequences (Baker & Marshall 1997). The control region of hornbills may also be useful in phylogenetic studies since it is characterised by three domains, each with different rates of change and levels of saturation. The transition-to-transversion ratio between species is greatest in Domain II. Therefore, this region would be most suited to phylogenetic analysis between species and possibly genera. The central domain is conserved and is probably not suitable for phylogenetic analysis. Furthermore, the between species transition-to-transversion ratio is not significantly different from that expected at saturation. This characteristic is probably the result of few transitions and low evolutionary rates within the central domain. Although this domain is not suitable for phylogenetic analysis it may provide support for deep phylogenetic nodes when combined with the more variable domain II. Finally, Domain I contains repeats of CSB-1 that occur in all eight of the Bucerotiformes considered. The population and phylogenetic utility of these repeats is clearly dependent on whether these motifs are continuously evolving even after they originated. Even though several repeat motifs occur in all eight of the Bucerotiformes, some are taxon-specific (Figure 4). Furthermore, the utility of these repeats is dependent on whether such taxon-specific motifs are consistently so. Delport (2000d Chapter 5) sequenced the 5' control region of five *T. e. rufirostris* individuals from allopatric and sympatric populations and determined that the taxon-specific motifs identified here were consistent across individuals. Domain I is not saturated and coupled with the taxon-specific repeat motifs is useful for phylogenetic analysis. However Delport (2000d Chapter 5) determined that Domain I is highly variable within species and therefore probably more useful for population analysis.

## Conclusions

We have determined that the bucerotiform mitochondrial DNA control region shares features with other avian control regions and exhibits some unique features. The unique features are the result of repeats of CSB-1 in the 5' end of hornbill control region. Similar repeats have been found in other bird species (Desjardins & Morais 1990, 1991, Quinn & Wilson 1993), however these repeat are not consecutive as in hornbills. Furthermore Quinn & Wilson (1993) have determined that the repeats in snowgoose, chicken and quail originated before the divergence of these lineages from one another. The hornbill repeats, however, have originated after the divergence of hornbills from chicken, snowgoose and quail. In addition these repeats are undergoing mutation and therefore would be useful in both population and phylogenetic studies.

# Chapter 5

Mitochondrial DNA gene flow between two morphologically distinct subspecies of African Redbilled Hornbill (*Tockus erythrorhynchus*) and implications for taxonomic status

"In the genetic programme, therefore, is written the result of all past reproductions, the collection of successes, since all traces of failures have disappeared."

Jacob 1973

**Abstract**

In order to determine the extent of gene flow between two subspecies of Redbilled Hornbill (*Tockus erythrorhynchus*), we sequenced 360 base pairs of cytochrome b and 627 base pairs of the 5' end of mitochondrial DNA (mtDNA) control region for 12 and 25 individuals respectively. These individuals were representative of allopatric and sympatric populations of *T. e. damarensis* and *T. e. rufirostris* and one outgroup, *T. monteiri*. The cytochrome b gene had only six variable sites between the populations of Redbilled Hornbill, whereas the control region was more variable (fifty-six variables sites in 627 base pairs). Therefore, we only present the population and phylogenetic analysis of the control region. The 5' end of the control region is similar to that of other bucerotiforms in that it is AT rich and comprises a series of 23 base pair repeats. We calculated sequence divergence ($D_{xy}$) and $\Phi_{st}$ values between sympatric and allopatric populations of both subspecies. Furthermore, we constructed a minimum spanning network of haplotypes and a maximum parsimony phylogenetic tree. All three analyses gave essentially the same results. Firstly, allopatric populations of both subspecies are significantly different. Secondly, in a zone of sympatry there appears to be asymmetric gene flow of *T. e. damarensis* haplotypes into *T. e. rufirostris*. Thirdly, sympatric *T. e. rufirostris* individuals are consistently different from those in allopatry, which may be the result of isolation by distance. Finally, there is reduced subspecific gene flow in sympatry and this observation, coupled with reduced heterospecific hatching success and morphological and behavioural differences, warrants each of these subspecies as separate species.

## Introduction

The avian mtDNA genome provides a useful tool for investigating evolutionary relationships both within and between species (Quinn 1997, Shields & Helm-Bychowski 1988). Specifically, the control region of the mtDNA genome has been used successfully in determining population structure within species and for identifying relationships among morphologically recognised subspecies (Baker & Marshall 1997, Quiestiau *et al.* 1998). Geographically based DNA analysis below the species level, termed phylogeography (Avise *et al.* 1987), retains a prominent role in evolutionary biology since it provides a window on the role of gene flow in the process of speciation. Furthermore, the absence or presence of gene flow has been imperative in defining species under all species concepts. The original proposers of the Biological Species Concept (Mayr 1963, Dobzhansky 1970) defined species only by the absence of gene flow. However, Ehrlich & Raven (1969) argued that a balance between gene flow and other evolutionary forces (such as natural selection) are important in defining species. Furthermore, Paterson's (1985) Recognition Species Concept defines species in terms of common fertilisation systems which essentially lead to gene flow within species. The first of several testable null hypotheses in the Cohesion Species Concept (Templeton 1989) is the test of whether the populations under study comprise a single evolutionary lineage (Templeton 1998) or a field of gene recombination. Finally, the Phylogenetic species concept (Cracraft 1989), defining species as lineages that have consistently diagnosable traits, is an analysis of pattern that is determined by the presence or absence of gene flow. Therefore the quantitative analysis of gene flow is imperative in any decision of taxonomic status.

The African Redbilled Hornbill subspecies complex (*Tockus erythrorhynchus*) comprises six morphological subspecies that are diagnosable on the basis of eye and circumorbital skin colour (Kemp 1995, Delport 2000a Chapter 2). These morphological characteristics are consistent within each of the subspecies (Kemp 1995, Delport 2000a Chapter 2, Delport 2000b Chapter 3) and may warrant each of the subspecies specific status. In this paper we survey mtDNA cytochrome b and control region variation within and between populations of two subspecies of African Redbilled Hornbill, *T. e. damarensis* and *T. e. rufirostris*. We aim to determine the genetic divergence between the two subspecies and whether the two Redbilled

Hornbill subspecies considered behave as a single random-mating population. Furthermore we discuss behavioural, morphological and breeding biology data collected previously (Delport 2000a Chapter 2, Delport 2000b Chapter 3) in light of the genetic data presented here and conclude that *T. e. rufirostris* and *T. e. damarensis* should be treated as separate species.

## Methods and Materials

*Study Area and sampling*

Two subspecies of Redbilled Hornbill, *T. e. damarensis* and *T. e. rufirostris*, hybridise (Delport 2000b Chapter 3) in a northern Namibian contact zone (19° 20' S, 17° 21' E). Therefore we collected samples from allopatric and sympatric populations of both subspecies. Blood samples were taken by puncture of the major patagial vein and mixed immediately in blood storage buffer (0.1M Tris-HCl, 0.04M EDTA·$Na_2$, 1.0M NaCl, 0.5% SDS), with a 1:10 ratio of blood to buffer. We removed females from nestboxes, bled and replaced them. A total of three *T. e. rufirostris* females and three hybrid females were bled from the contact zone in northern Namibia. Blood samples were stored at -4°C. In addition to these blood samples we collected four *T. e. rufirostris*, four *T. e. damarensis* and one hybrid male from the zone of sympatry. We skinned these males and removed heart, liver and skeletal muscle for molecular analyses. All tissues were stored at -20° C. Skins were then deposited with the Namibian Natural History Museum. In addition to the samples collected in sympatry we obtained blood samples from four allopatric *T. e. damarensis* females at the Daan Viljoen Nature Reserve, Windhoek (22° 32' S, 16° 58' E) and from four allopatric *T. e. rufirostris* males and one female in the Northern Province, South Africa (23° 00' S, 28° 35' E). These allopatric samples were used to identify pure *T. e. damarensis* and pure *T. e. rufirostris* haplotypes. Furthermore. we obtained one *T. monteiri* blood sample, from Daan Viljoen Game Reserve, for outgroup analysis.

*DNA extraction, PCR amplification and sequencing*

The genomic DNA extraction procedure used is described elsewhere (Delport 2000c Chapter 4). We amplified the 5' end of cytochrome b and the 5' domain of the control region in polymerase chain reactions (PCR). The PCR reaction conditions used are those described in Delport (2000c Chapter 4). We used the primers L14841 (Kocher

*et al.* 1989) and H15499 (Avise *et al.* 1994) to amplify the 5' end of cytochrome b. The primers HBDL2H (5' ATACGTCTGGCATATCCG 3'), that anneals 808 bp from the 5' end of the control region, and L16746 (Wenink *et al.* 1994), that anneals in tRNA-Glu, were used to amplify the 5' end of the control region. However, amplification of allopatric *T. e. rufirostris* individuals with these primers was non-specific. Therefore, we used Delport's (2000c Chapter 4) HBND6L primer in combination with HBDL2H in the allopatric *T. e. rufirostris* PCR reactions. The PCR products were purified using a High Pure™ PCR Product Purification kit (Boehringer Mannheim), according to the manufacturer's instructions. Dye-terminator cycle sequencing was performed, using primers L14841 and H15499 for cytochrome b and HBGlu-L (Delport 2000c Chapter 4) and HBDL2H for the control region, with the BigDye DNA Ready reaction sequencing kit (Applied Biosystems, California) in a Geneamp® PCR System 9700 (Applied Biosystems, California). Thereafter, nucleotide sequences were determined through electrophoresis on an ABI 377 automated sequencer (Applied Biosystems, California).

*Sequence analysis*

Heavy and light strand sequences for both cytochrome b and control region were imported into Sequence Navigator version 1.0.1 (Applied Biosystems, California), where they were proof read. Consensus sequences were aligned using CLUSTAL W version 1.74 (Higgins *et al.* 1992). Aligned sequences were imported into both MEGA version 2.0 (Kumar *et al.* 2000) and PAUP version 4.0 (Swofford 1999). We calculated control region sequence divergence between sympatric and allopatric populations as Nei's (1987) $D_{xy}$, the average number of nucleotide substitutions per site between populations, using MEGA (Kumar *et al.* 2000). Furthermore, we used Excoffier's (1992) program WINAMOVA to calculate $\Phi_{st}$. Statistical support of between-population $\Phi_{st}$ values were calculated in WINAMOVA with 1000 permutations. Phylogenetic trees were drawn (in PAUP) using the heuristic maximum parsimony search criterion and the consensus topology was determined by 50% majority rule. Statistical support of the consensus topology was calculated using 1000 bootstrap replicates (Felsenstein 1985). Finally a minimum spanning network of haplotypes was determined with MINSPNET (Excoffier 1993).

## Results

*Cytochrome b and control region characteristics*

A total of 360 base pairs of cytochrome b and 627 base pairs of the 5' end of the control region were sequenced for each of twelve and twenty-five individuals respectively. Cytochrome b mean base frequencies (A = 25.3, C = 33.7, G = 16.5 & T = 24.5) are similar to that of cytochrome b in other bird species (Moore & DeFilippis 1997). Mean base frequencies (A = 34.5, C = 17.6, G = 14.3 & T = 33.6) of the twenty-five control region sequences are similar to that of other bucerotiform control regions sequenced (Delport 2000c Chapter 4). The mean transition-to-tranversion ratios within Redbilled Hornbills are 2.17 for cytochrome b and 6.0 for the control region, which are both far greater than that of 0.5 expected at saturation (Holmquist 1983). Only six of the 360 cytochrome b sites were variable whereas fifty-six of the 627 control region sites were variable between the Redbilled Hornbills sequenced (Figure 1). The fifty-six control region sites defined twenty distinct mtDNA haplotypes from the twenty-four Redbilled Hornbills sequenced (Figure 1). Delport (2000c Chapter 4) identified 13 repeats of 23 base pairs in the 5' end of the control regions of bucerotiforms and determined that these repeats contain phylogenetic signal. Furthermore, some mutations within the repeats are consistent within particular populations (Figure 1) and therefore are informative in intraspecific analyses.

*Population and phylogenetic analyses*

Sequence divergence between populations is generally greater than that within populations (Table 1a). However both hybrids and sympatric *T. e. rufirostris* individuals have within population $D_{xy}$ values that approximate that between populations. Furthermore, sequence divergence between allopatric and sympatric populations of *T. e. damarensis* is low and approximates that within each of these populations. Finally, allopatric *T. e. rufirostris* shows the greatest sequence divergences from all other populations, including sympatric *T. e. rufirostris*. The $\Phi_{st}$ statistics represent the same scenario with half of the pairwise comparisons significantly different (Table 1b). The non-significant sequence divergences occur

Nucleotide positions

```
                  1222333333333444444444444444555555555555555555556666666
                  6479001446778001134444557999111122344556666667770245555
                  1814457083346697922378058356568919719151356792569175345 6

h 1   (ad)       GAGATAAAAATAATGATAGATTCAACCTTATCGCCCGTCGACACCCTCCTTGACCC
h 2   (ad)       .............................................C.........
h 3   (sd)       .........G.....G....C..........TA............C.........
h 4   (sr,h)     ..AG.....G.....G................T............C.........
h 5   (sd)       .........G.....G.G..C....TT.C...A............CT..C.....
h 6   (ad)       .........G.....G.G..C....TT.C...A............CT..C....T
h 7   (sr)       .........G.....G.G..C....TT.C.C.A.....T.......CTT.C.....
h 8   (h)        ..A...G..G.....G....C....TT.C...A........T....CT..C.....
h 9   (h)        ..A...G..G.....G....C....TT.C...A............CT..C.....
h 10  (sd)       .......G.......G.........T.....TA............C...C.....
h 11  (sr)       ...............G.........T.....TA..........T..CT..C.....
h 12  (sr)       .GA....GG......G..T..AT..T.C...TA..T..T...G..TCT...A....
h 13  (sr)       ..AG...G....G.A..GT..AT.GT.....TA.....T.......C...C.....
h 14  (h)        ..AG...G....G.A..GT..AT..T.....TA.....T.G.....C...C.....
h 15  (sr)       ..AG...G....G.A..GT..AT.GT...G.TA.....T...........C.....
h 16  (ar)       A...GT.GG.GT...G...T.ATG.T.C....A.T.AC.AGTG.T.C..A....T.
h 17  (ar)       A...GTGGG.GT...G...T.ATG.T.C....A.T.AC.AGTG.T.C..A....T.
h 18  (ar)       AGA.GT.GG.GT.C.G...T.AT.........A.T.AC.AGTG.T.C..A..G.T.
h 19  (ar)       A...GT.GG.GT...GC..T.ATG..TC....A...AC.AGTG.T.C..A.A..T.
h 20  (ar)       A...GT.GG.GT...GC..T.AT....C....A.T.AC.AGTG.T.C..ACA.TT.
```

**Figure 1:** Variable sites of the mitochondrial DNA control region haplotypes. Nucleotide positons for control region haplotypes are as in *Bucorvus leadbeateri* mtDNA control region sequence (Delport 2000b). Identity with haplotype 1 is represented by dots (.) and insertions/deletions by dashes (-). Taxonomic designations in parenthesis are as follows: ad = allopatric *T. e. damarensis*, sd = sympatric *T. e. damarensis*, h = hybrid, sr = sympatric *T. e. rufirostris* and ar = allopatric *T. e. rufirostris*. Allopatric *T. e. r.* haplotypes show that some mutations are specific to populations and are consistent within allopatric *T. e. rufirostris* (bold).

**Table 1:** Mitochondrial DNA control region sequence divergence between five populations of *Tockus erythrorhynchus*. Divergences were estimated using a) the average number of nucleotide substitutions per site between populations (Nei's [1987] $D_{xy}$ with Tamura-Nei correction) and b) $\Phi_{st}$ of Excoffier et al [1992]. Within population divergences were estimated using Nei's [1987] $D_{xy}$ (with Tamura-Nei correction) and are in boldtype in a). Sample sizes for each pairwise comparison are shown above the diagonal in a). The probability that a random distance (1000 permutations) is different from the observed distance ($\Phi_{st}$) for each pairwise comparison is shown above the diagonal in b). Significant probabilities are indicated in bold. Taxonomic groups are *T. e. d* (a) = allopatric *T. e. damarensis*, *T. e. d* (s) = sympatric *T. e. damarensis*, *T. e. r* (a) = allopatric *T. e. rufirostris*, *T. e. r* (s) - sympatric *T. e. rufirostris* and hybrids. Cytochrome b divergences were not calculated since 360 bp yielded low variation between individual Redbilled Hornbills.

| a) | 1. | 2. | 3. | 4. | 5. |
|---|---|---|---|---|---|
| 1. *T. e. d* (a) | **0.010** | 8 | 9 | 11 | 8 |
| 2. *T. e. d* (s) | 0.011 | **0.008** | 9 | 11 | 8 |
| 3. *T. e. r* (a) | 0.044 | 0.042 | **0.010** | 12 | 9 |
| 4. *T. e. r* (s) | 0.018 | 0.019 | 0.049 | **0.022** | 11 |
| 5. hybrids | 0.018 | 0.016 | 0.046 | 0.021 | **0.020** |

| b) | 1. | 2. | 3. | 4. | 5. |
|---|---|---|---|---|---|
| 1. *T. e. d* (a) | | 0.196 | **0.000** | 0.072 | 0.098 |
| 2. *T. e. d* (s) | 0.1852 | | **0.000** | **0.000** | 0.310 |
| 3. *T. e. r* (a) | 0.8021 | 0.8055 | | **0.000** | **0.000** |
| 4. *T. e. r* (s) | 0.2611 | 0.2188 | 0.6857 | | 0.333 |
| 5. hybrids | 0.1550 | 0.0614 | 0.7065 | -0.0120 | |

between (i) allopatric and sympatric *T. e. damarensis*, (ii) allopatric *T. e. damarensis* and sympatric *T. e. rufirostris*, (iii) allopatric *T. e. damarensis* and hybrids, (iv) sympatric *T. e. damarensis* and hybrids and (v) sympatric *T. e. rufirostris* and hybrids. The minimum spanning network of control region haplotypes shows some geographical subdivision with allopatric *T. e. rufirostris* as a separate group (Figure 2). Allopatric *T. e. damarensis* individuals are separated from one another by one to fourteen mutations, with interspersed sympatric *T. e. damarensis*, sympatric *T. e. rufirostris* and hybrid haplotypes. Furthermore, some sympatric *T. e. rufirostris* haplotypes are similar to both allopatric and sympatric *T. e. damarensis* haplotypes, whereas others are considerably different. However, no sympatric *T. e. rufirostris* haplotypes show close relation to allopatric *T. e. rufirostris* haplotypes.

The phylogenetic analysis showed the same phenomena as the population and haplotype analyses (Figure 3). Firstly, the allopatric *T. e. rufirostris* monophyletic clade (with strong bootstrap support) is clearly divergent from all others. Secondly, another clade with bootstrap support (66%) comprises allopatric and sympatric *T. e. damarensis* individuals, sympatric *T. e. rufirostris* individuals and hybrids. This second clade is further subdivided. One subclade comprises sympatric *T. e. rufirostris* individuals and one hybrid and another comprises only allopatric *T. e. damarensis* individuals. The remaining sympatric *T. e. rufirostris*, sympatric *T. e. damarensis* and allopatric *T. e. damarensis* individuals are interspersed within the second major clade.

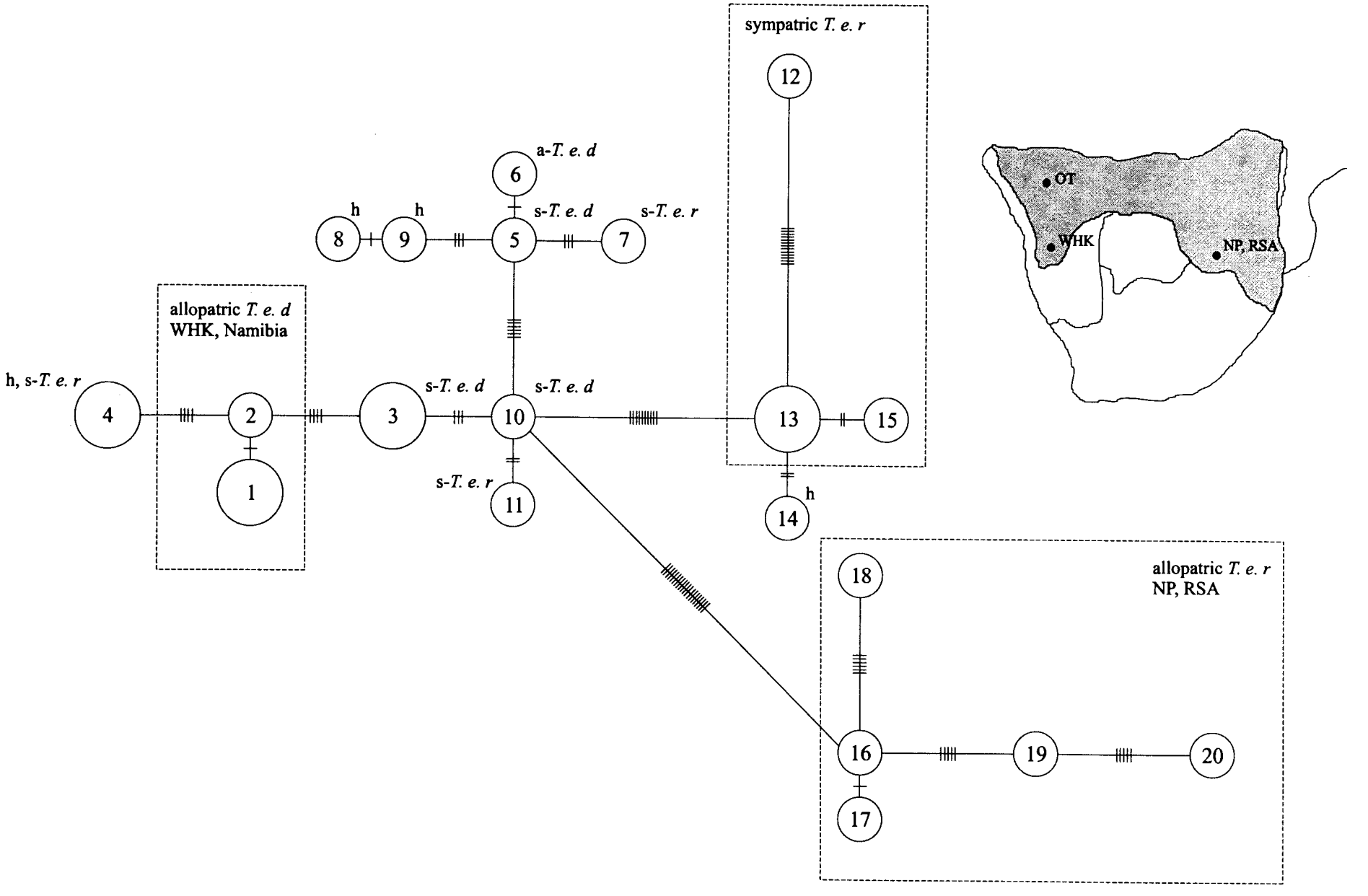



**Figure 2:** Minimum spanning network of control region haplotypes and distribution of *Tockus erythrorhynchus* in southern Africa. Haplotype numbers are as in Figure 1. s-*T. e. r* = sympatric *T. e. rufirostris*, s-*T. e. d* = sympatric *T. e. damarensis*, a-*T. e. d* = allopatric *T. e. damarensis*, h = hybrid, NP = Northern Province, RSA = Republic of South Africa, WHK = Windhoek, OT = Otavi (zone of sympatry).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

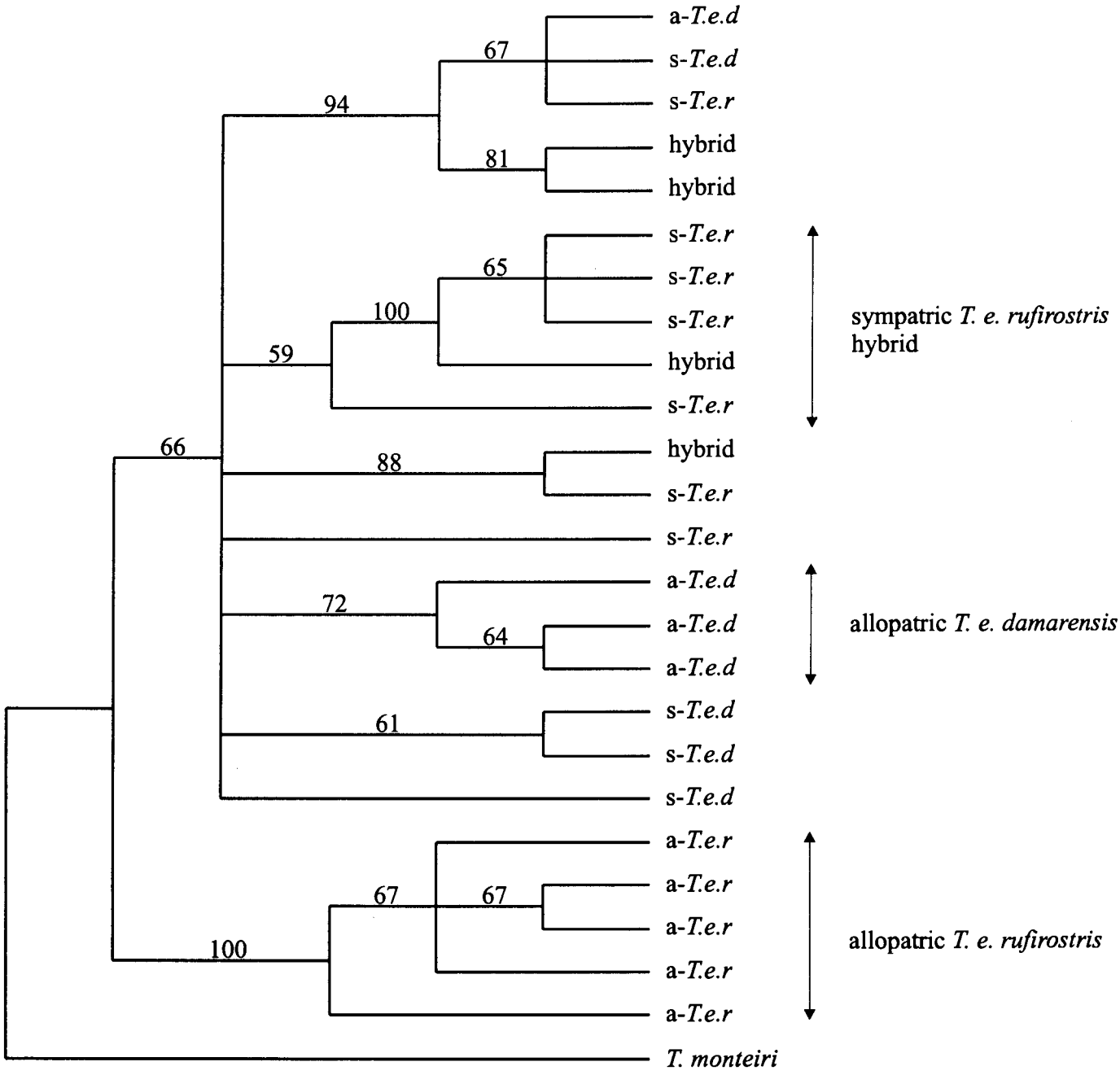



**Figure 3:**Maximum parsimony phylogenetic tree constructed in PAUP version 4 (Swofford 1999). The tree shown is the 50% majority rule bootstrap topology of eighty-two most parsimonious trees. The strict consensus tree had essentially the same topology. All branches with bootstrap values (1000 replicates) below 50 are collapsed. Taxonomic designations are as follows: a-*T.e.r* = allopatric *T. e. rufirostris*, s-*T.e.r* = sympatric *T. e. rufirostris*, s-*T.e.d* = sympatric *T. e. damarensis* and a-*T.e.d* = allopatric *T. e. damarensis*. Phylogenetic analysis of 360 bp of cytochrome b yielded a topology that was less resolved. However the allopatric *T. e. rufirostris* clade also had strong bootstrap support in that analysis.

## Discussion

*Gene flow and taxonomic implications*

As we would expect, gene flow between allopatric and sympatric *T. e. damarensis* is high and these populations are not significantly different. However gene flow between allopatric and sympatric *T. e. rufirostris* is considerably lower. This contrast may be the result of two processes: asymmetric mtDNA gene flow in the contact zone or isolation by distance between the sympatric and allopatric populations of *T. e. rufirostris*. Asymmetric gene flow is well documented in a number of hybrid zones (Barton 1986, Lamp & Avise 1986, Sperling & Spence 1991, Parsons, Olson & Braun 1993) and may occur in the Redbilled Hornbill hybrid zone. Firstly, the sympatric *T. e. rufirostris* individuals have greater within population divergences than that of allopatric *T. e. rufirostris*, whereas divergences within allopatric and sympatric *T. e. damarensis* populations sequences are equivalent (Table 1a). This anomaly suggests that hybridisation has resulted in mtDNA gene flow from *T. e. damarensis* into *T. e. rufirostris* but not *vice versa*. Delport (2000b Chapter 3) provides support for this notion since mating in the contact zone is non-random, there is a minority of heterospecific pairs and the female *T. e. damarensis* – male *T. e. rufirostris* hybrid pair occurs more frequently than the opposite combination. Given that mtDNA is maternally inherited such asymmetric pair combinations may lead to asymmetric gene flow of mtDNA. Furthermore, the $\Phi_{st}$ statistics represent a similar scenario with no significant differences between allopatric *T. e. damarensis* and each of the following: sympatric *T. e. damarensis*, sympatric *T. e. rufirostris* and hybrids (Table 1b). In addition sympatric *T. e. damarensis* and sympatric *T. e. rufirostris* individuals are not significantly different from hybrids (Table 1b). However, sympatric *T. e. damarensis* and sympatric *T. e. rufirostris* individuals do exhibit significant differences. This result is not expected under asymmetric introgression and therefore the reduced gene flow between sympatric and allopatric *T. e. rufirostris* populations may also be the result of isolation by distance.

In addition to the sympatric *T. e. rufirostris* individuals that have *T. e. damarensis* haplotypes, there are some unique sympatric *T. e. rufirostris* haplotypes (Figure 2, Figure 3). These sympatric *T. e. rufirostris* haplotypes have most likely dispersed from a local allopatric *T. e. rufirostris* population. Therefore, the apparent lack of

gene flow between allopatric and sympatric *T. e. rufirostris* populations may also be the result of isolation by distance and not only asymmetric introgression. Both of the above hypotheses receive support from the haplotype and phylogenetic trees, since (i) most sympatric *T. e. rufirostris* and *T. e. damarensis* individuals are similar to allopatric *T. e. damarensis* and (ii) since there are sympatric *T. e. rufirostris* individuals that are distinct from other individuals that occur in sympatry. Only increased sampling (i) of sympatric individuals of both subspecies and hybrids and (ii) of allopatric populations of each subspecies flanking the hybrid zone can determine whether the observed pattern of population differentiation is the result of one or both of the above processes. In addition, the use of a biparentally inherited molecular marker, such as nuclear microsatellite repeats, would contribute significantly to the understanding of this hybrid zone.

Gene flow between allopatric populations of *T. e. rufirostris* and *T. e. damarensis* is limited, although this result is expected on the basis of distance between the allopatric population sample localities. However, there are lower levels of gene flow, and greater sequence divergence, between allopatric *T. e. damarensis* and sympatric *T. e. rufirostris* than between allopatric and sympatric *T. e. damarensis* populations. This observation suggests there is some barrier to gene flow between the subspecies, since sympatric populations of both are separated from allopatric *T. e. damarensis* by an equivalent distance. Furthermore, Delport (2000b Chapter 3) has determined that there are consistent morphological differences between allopatric populations of these two subspecies. Diagnosability on the grounds of both morphological and molecular data provide support for the assignment of separate species under the Phylogenetic Species Concept (Cracraft 1989). Other species concepts such as the Biological Species Concept (Mayr 1963, Dobzhansky 1970), the Recognition Species Concept (Paterson 1985) and the Cohesion Species Concept (Templeton 1989) define species in terms of processes and not patterns as in the Phylogenetic Species Concept (Cracraft 1989). Delport (2000b Chapter 3) determined that allopatric populations of *T. e. damarensis* and *T. e. rufirostris* have vocalisations that are consistently distinct from one another. These vocalisations may therefore be a conspecific signal that has the fortuitous consequence of prevention of mating between heterospecifics that results in reduced gene flow between the two subspecies in the zone of sympatry. Species-specific vocalisations would provide support for the identification of

independent species under both the Cohesion Species Concept (Templeton 1989) and the Recognition Species Concept (Paterson 1985), yet the Biological Species Concept further requires that there be no mating between heterospecifics. The two subspecies of Redbilled Hornbill considered hybridise. However it is well documented that several avian species hybridise (Grant & Grant 1992). Ehrlich & Raven's (1969) modification of the Biological Species Concept includes a balance between gene flow and other evolutionary forces (e.g. selection) in the definition of a species. One of these evolutionary forces or effects of hybridisation in the Redbilled Hornbills is the reduced fitness of heterospecific pairs as determined by Delport 2000b (Chapter 3). Therefore, in the context of Ehrlich & Raven's (1969) version of the Biological Species Concept, the two subspecies of Redbilled Hornbill would be considered independent species. Crowe (1999) has proposed that consensus between several data sources, in a Multifaceted Concept of Species, is sufficient to determine species status. Therefore, subspecies that have morphological, behavioural and genetic differences combined with reduced heterospecific gene flow and hybridisation incompatability are true species under any of the above species definitions.

# Chapter 6

## Conclusions

"By continous summation of small useful variations through many generations definite specific characters would in time be achieved and new species arise"

Smuts 1926

In this thesis I have presented and discussed results which indicate that *Tockus erythrorhynchus damarensis*, the Damaraland Redbilled hornbill should be considered a separate species from *Tockus erythrorhynchus rufirostris*, the southern African Redbilled Hornbill. Several lines of evidence indicate that these subspecies deserve to be identified as independent species. Firstly, the two subspecies have diagnosable morphological characteristics that are effectively fixed within allopatric populations (Kemp 1995, Delport 2000b Chapter 3). Secondly, although the two subspecies hybridise, they do so non-randomly and the male *T. e. rufirostris* – female *T. e. damarensis* pair combination occurs more frequently than vice versa (Delport 2000b Chapter 3). In addition to this asymmetric pair formation, heterospecific pairs have significantly lower hatching success than homospecific pairs (Delport 2000b Chapter 3). Finally, there is some barrier to gene flow in the zone of sympatry (Delport 2000d Chapter 5) which may present itself as a failure in communication between heterospecifics or through the reduced fitness of heterospecific pairs. Although these mechanisms have not been elucidated completely, the patterns used to infer these processes are evident in the Redbilled Hornbill hybrid zone.

Several questions remain to be addressed in the Redbilled Hornbill hybrid zone. Firstly, it would be interesting to investigate the concept of asymmetric introgression of *T. e. damarensis* haplotypes into sympatric *T. e. rufirostris* individuals. This asymmetrical gene flow is supported by the pair formation since there are more female *T. e. damarensis* – male *T. e. rufirostris* hybrid pairs than *vice versa*. However, to be certain of asymmetric gene flow one would need to sample extensively both within the hybrid zone and at its periphery. Furthermore, a nuclear marker would be useful to determine whether the apparent asymmetric mtDNA gene flow is balanced by nuclear DNA gene flow in the opposite direction. Secondly, it would be interesting to determine whether the asymmetric pattern of pair formation is the result of mate choice or the result of a breakdown in communication of one of the two possible pair combinations. Such research would require some carefully designed experiments in the zone of sympatry. Finally, the asymmetric pattern of call inheritance, where most sympatric individuals have *T. e. damarensis* calls, would be an interesting aspect to investigate further within the zone of sympatry. Again some carefully designed and executed experiments within the contact zone are required.

In addition to the research in the contact zone, some interesting results which concern the evolution of the bucerotiform mtDNA control region have been discussed. The bucerotiform control region has characteristics that are homologous to other avian species, yet it also exhibits unique characteristics. The next most obvious step would be to sequence the control region of closely related orders, such as the coraciformes, to determine where the unique control regions of hornbills have originated. In addition, such research would contribute to the existing knowledge of the structure and function of the mtDNA control region.

Finally, the most obvious extension of this research would be to include the remaining four subspecies of African Redbilled Hornbill in a phylogenetic analysis. Furthermore the research on the Damaraland Redbilled Hornbill (*T. e. damarensis*) and the southern African Redbilled Hornbill (*T. e. rufirostris*) has strong implications for the taxonomic status of the remaining four subspecies. Obviously, it would be confusing to raise two of the subspecies to the species level and leave the remaining four since one now begs the question "to which species do these remaining subspecies then belong?". It would be ideal to investigate each of the contact zones between the subspecies as I have done with *T. e. damarensis* and *T. e. rufirostris*. However, given the localities of these putative contact zones I am unable to do so. Now is the time for extrapolation. I believe if I am able to generate a molecular phylogeny of the African Redbilled Hornbills that indicates there are diagnosable molecular characteristics that differ consistently between the subspecies then these subspecies deserve specific status.

In conclusion, the assignment of subspecies as separate species has imperative conservation implications. Firstly, the Damaraland Redbilled Hornbill now joins the other 10 Namibian endemics (Jarvis & Robertson 1997) and should be included in the endemic monitoring program. Furthermore, the distribution of the Damaraland Redbilled Hornbill mirrors that of seven of the Namibian endemics (Hartlaub's Francolin *Francolinus hartlaubi*, Rüppel's Parrot *Poicephalus rueppellii*, Violet Woodhoopoe *Phoeniculus damarensis*, Monteiro's Hornbill *Tockus monteiri*, Carp's Black Tit *Parus carpi*, Rockrunner *Achaetops pycnopygius* and Whitetailed Shrike *Lanioturdus torquatus*. Perhaps a comparative study would elucidate a common speciation mechanism in this diverse array of taxa. Secondly, the Senegambian

Redbilled Hornbill (*T. e. kempi*), the Tanzanian Redbilled Hornbill and Samburu National Park Redbilled Hornbill have limited distributions. Therefore, the population sizes of each of these 'species' would need to be determined if we are to conserve biodiversity.

# References

AQUADRO, C. F., & GREENBERG, B. D. 1983. Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. Genetics 103: 287-312.

ARNOLD, M. L. & HODGES, S. A. 1995. Are natural hybrids fit or unfit relative to their parents? TREE 10: 67-71.

AVISE, J. C., NELSON, W. S. & SIBLEY, C. S. 1994. DNA sequence support for a close phylogenetic relationship between some storks and New World vultures. Proceedings of the National Academy of Sciences, USA 91: 5173-5177.

AVISE, J. C., ARNOLD, J., BALL, R. M. Jr, BERMINGHAM, E., LAMB, T., NEIGEL, J. E., REEB, C. A. & SAUNDERS, N. C. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annual Review of Ecology and Systematics 18: 489-522.

BAKER, A. J. & MARSHALL, H. D. 1997. Mitochondrial control region sequences as tools for understanding evolution. In D. P. Mindell (ed) Avian Molecular Evolution and Systematics. Academic Press, San Diego.

BARTON, N. H. & GALE, K. S. 1993. Genetic analysis of hybrid zones. In R. G. Harrison (ed) Hybrid zones and the evolutionary process. Oxford University Press, New York.

BARTON, N. H. & HEWITT, G. M. 1985. Analysis of hybrid zones. Annual Review of Ecology and Systematics 16: 113-148.

BARTON, N. H. & HEWITT, G. M. 1989. Adaptation, speciation and hybrid zones. Nature 341: 497-503.

BARTON, N. H. 1979. The dynamics of hybrid zones. Heredity 43: 333-339.

BARTON, N. H. 1986. The effect of linkage and density-dependent regulation on gene flow. Heredity 57: 415-426.

BRETAGNOLLE, V. & GENEVOIS, F. 1997. Geographic variation in the call of the Blue Petrel: effects of sex and geographic scale. The Condor, 99: 985-989.

BROWN, G. G., GADALETA, G., PEPE, G., SACCONE, C. & SBISÀ, E. 1986. Structural conservation and variation in the D-loop-containing region of vertebrate mitochondrial DNA. Journal of Molecular Biology 192: 503-511.

BROWN, W. M., GEORGE, M. & WILSON, A. C. 1979. Rapid evolution of animal mitochondrial DNA. Proceedings of the National Academy of Sciences USA 76: 1967-1971.

BUTLIN, R. 1989. Reinforcement of premating isolation. In D. Otte & J. A. Endler (eds) Speciation and Its Consequences: 3-27. Sinauer Associates. Sunderland, Massachusetts.

BYERS, B. E. 1996. Geographic variation of song form within and among Chestnut-sided Warbler populations. Auk 113: 288-299.

CANN, R. L., BROWN, W. M. & WILSON, A. C. 1984. Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. Genetics 106: 479-499.

CHAPMAN, F. M. 1892. A preliminary study of the grackles of the subgenus Quiscalus. Bulletin of the American Museum of Natural History 4: 1-19.

CLAYTON, D. A. 1991. Replication and transcription of vertebrate mitochondrial DNA. Annual Review of Cell Biology 7: 453-478.

CRACRAFT, J. 1989. Speciation and its ontology: The empirical consequences of alternative species concepts for understanding patterns and processes of

differentiation. In D. Otte & J. A. Endler (eds) Speciation and Its Consequences: 3-27. Sinauer Associates. Sunderland, Massachusetts.

CROWE, T. M. 1999. Species as multifaceted entities. In N. J. Adams & R. H. Slotow (eds). Proceeding of the 22nd International Ornithology Congress, Durban: 1490-1495. BirdLife South Africa, Johannesburg.

DARWIN, C. 1859. On the Origin of the Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life. John Murray, London.

DE QUEIROZ, A. & WIMBERGER, P.H. 1993. The usefulness of behavior for phylogeny estimation: levels of homoplasy in behavioral and morphological characters. Evolution 47: 46-60.

DELBRÜCK 1966. In B. Lewin. 1994. Genes V. Oxford University Press, New York.

DELPORT, W. 1999. Mate Recognition among Red-billed Hornbills. Africa Birds and Birding 4(5): 26-27.

DELPORT, W. 2000a. Geographic call variation between subspecies of the African Redbilled Hornbills (*Tockus erythrorhynchus* subspecies). MSc Thesis Chapter 2: 6-22. University of Pretoria, Pretoria.

DELPORT, W. 2000b. The structure of an African Redbilled Hornbill hybrid zone revealed by morphological, behavioural and breeding biology data. MSc Thesis Chapter 3: 23-51. University of Pretoria, Pretoria.

DELPORT, W. 2000c. Characterisation and evolution of the mitochondrial DNA control region in Hornbills (Bucerotiformes). MSc Thesis Chapter 4: 52-74. University of Pretoria, Pretoria.

DELPORT, W. 2000d. Mitochondrial DNA gene flow between two morphologically distinct subspecies of African Redbilled Hornbill (*Tockus erythrorhynchus*) and

implications for taxonomic status. MSc Thesis Chapter 5: 75-88. University of Pretoria, Pretoria.

DESJARDINS, P. & MORAIS, R. 1990. Sequence and gene organisation of the chicken mitochondrial genome: A novel gene order in higher vertebrates. Journal of Molecular Biology 212: 599-634.

DESJARDINS, P. & MORAIS, R. 1991. Nucleotide sequence and evolution of coding and noncoding regions of a quail mitochondrial genome. Journal of Molecular Evolution 32: 153-161.

DIAMOND, J. 1998. Geographic variation in vocalisations of the White-eye superspecies *Zosterops* (Griseotinctus) in the New Georgia group. Emu, 98: 70-74.

DOBZHANSKY, Th. 1940. Speciation as a stage in evolutionary divergence. American Naturalist 74: 312-321.

DOBZHANSKY, Th. 1970. Genetics of the Evolutionary Process. Columbia University Press. New York.

EHRLICH, P. R. & RAVEN, P. H. 1969. Differentiation of populations. Science 165: 1228-1232.

ENDLER, J. A. 1977. Geographic Variation, Speciation and Clines. Princeton University Press. Princeton.

EXCOFFIER, 1992. WINAMOVA 1.04. Analysis of Molecular Variance for Windows. Distributed by L. Excoffier, University of Geneva.

EXCOFFIER, 1993. MINSPNET. Distributed by L. Excoffier, University of Geneva.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791.

FERGUSON, J. W. H. 1999. The significance of mate selection and mate recognition in speciation. In N. J. Adams & R. H. Slotow (eds). Proceeding of the 22nd International Ornithology Congress, Durban: 1496-1504. BirdLife South Africa, Johannesburg.

FORAN, D. R., HIXSON, J. E. & BROWN, W. M. 1988. Comparisons of ape and human sequences that regulate mitochondrial DNA transcription and D-loop DNA synthesis. Nucleic Acids Research 16: 5841-5861.

FREELAND, J. R. & BOAG, P. T. 1999. Phylogenetics of Darwin's finches: paraphyly in the tree-finches, and two divergent lineages in the warbler finch. Auk 116: 577-588.

GILL, F. B. 1997. Local cytonuclear extinction of the Golden-Winged Warbler. Evolution 51: 519-525.

GILL, F. B. 1998. Hybridization in birds. Auk 115: 281-283.

GRANT, P. G. & GRANT, R. 1992. Hybridization of bird species. Science 256: 193-197.

GRANT, T. 1999. Characterisation of the mitochondrial DNA control region of Namaqua Sandgrouse. Honours thesis. University of Pretoria, Pretoria.

HALDANE, J. B. S. 1954. An exact test for randomness of mating. Journal of Genetics 52: 631-635.

HARRISON, R. G. & RAND, D. M. 1989. Mosaic hybrid zones and the nature of species boundaries. In D. Otte & J. A. Endler (eds) Speciation and Its Consequences: 3-27. Sinauer Associates. Sunderland, Massachusetts.

HEWITT, G. M. 1988. Hybrid zones - natural laboratories for evolutionary studies.

TREE 3: 158-167.

HIGGINS, D. G., FUCHS, R. & BLEASBY, A. 1992. CLUSTAL: a new multiple sequence alignment program. Computational Applied Biosciences. 8: 189-191.

HOFACKER, I.L., STADLER, P. F., BONHOEFFER, S. & FONTANA, W. 1995. The Vienna RNA package: a comprehensive package for RNA secondary structure calculation and analysis for Windows 95/NT 3.51. ftp.itc.univie.ac.at.

HOLMQUIST, R. 1983. Transitions and transversions in evolutionary descent: an approach to understanding. Journal of Molecular Evolution 19: 134-144.

HOYT, D. 1979. Practical methods of estimating volume and fresh weight of birds' eggs. Auk 96: 73-77.

JACOB, F. 1973. In B. Lewin. 1994. Genes V. Oxford University Press, New York.

JARVIS, A. & ROBERTSON, T. 1997. Endemic birds of Namibia: evaluating their status and mapping biodiversity hotspots. Ministry of Environment and Tourism Research Discussion Paper Number 14. Directorate of Environmental Affairs, Windhoek, Namibia.

KEMP, A. C. 1976. A study of the ecology, behaviour and systematics of *Tockus* hornbills (Aves: Bucerotidae). Memoirs of the Transvaal Musuem 20.

KEMP, A. C. 1995. Bird Families of the World: The Hornbills Bucerotiformes. Oxford University Press, New York.

KIMBALL, R. T., BRAUN, E. L., ZWARTJES, P. W., CROWE, T. M. & LIGON, J. D. 1999. A molecular phylogeny of the pheasants and patridges suggests that these lineages are not monophyletic. Molecular Phylogenetics and Evolution 11: 38-54.

KOCHER, T. D., THOMAS, W. K., MEYER, A., EDWARDS, S. V., PÄÄBO, S., VILLABLANCA, F. X. & WILSON, A. C. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences, USA 86: 6196-6200.

KROODSMA, D. E. & BAYLIS, J. R. 1982. A world survey of vocal learning in birds. In D. E. Kroodsma & E. H. Miller (eds) Acoustic Communication in Birds volume 2: 311-337. Academic Press. New York.

KROODSMA, D. E., BAKER, M. C., BAPTISTA, L. F. & PETRINOVICH, L. 1984. Vocal "dialects" in Nuttall's White-crowned Sparrow. Current Ornithology 2: 103-133.

KROODSMA, D. E., BYERS, B. E., HALKIN, S. L., HILL, C., MINIS, D., BOLSINGER, J. R., DAWSON, J., DONELAN, E., FARRINGTON, J., GILL, F. B., HOULIHAN, P., INNES, D., KELLER, G., MACAULAY, L., MARANTZ, C. A., ORITZ, J., STODDARD, P. K. & WILDA, K. 1999. Geographic variation in Black-Capped Chickadee songs and singing behavior. The Auk, 116: 387-402.

KUMAR, S., TAMURA, K. & NEI, M. 2000. MEGA: Molecular Evolutionary Genetics Analysis, version 2.0. Pennsylvania State University, University Park, PA.

LAMB, T. & AVISE, J. C. 1986. Directional introgression of mitochondrial DNA in a hybrid population of tree frogs: the influence of mating behavior. Proceedings of the National Academy of Sciences, USA 83: 2526-2530.

LANGTIMM, C. A. & DEWSBURY, D. A. 1991. Phylogeny and evolution of rodent copulatory behaviour. Animal Behaviour 41: 217-225.

LANSMAN, R. A., AVISE, J. C. & HUETTEL, M. D. 1983. Critical experimental test of the possibility of 'paternal leakage' of mitochondrial DNA. Proceedings of the National Academy of Sciences, USA 80: 1969-1971.

LANSMAN, R. A., SHADE, R. O., SHAPIRA, J. F. & AVISE, J. C. 1981. The use of restriction endonucleases to measure mitochondrial sequence relatedness in natural populations III: Techniques and potential applications. Journal of Molecular Evolution 17: 214-226.

LANYON, W. E. 1969. Vocal characters and avian systematics. In R. A. Hinde (ed) Bird Vocalizations: their relations to current problems in biology and psychology: 291-310. Cambridge University Press, Cambridge.

LINDELL, C. 1998. Limited geographic variation in the vocalizations of a neotropical Furnariid, Synallaxis albescens. Wilson Bulletin 110: 368-374.

LOUIS, E. J. & DEMPSTER, E. R. 1987. An exact test for Hardy-Weinberg and Multiple Alleles. Biometrics 43: 805-811.

MARLER, P. & TAMURA, M. 1962. Song "dialects" in three populations of White-crowned sparrows. Condor 64: 368-377.

MARSHALL, H. D. & BAKER, A. J. 1997. Structural conservation and variation in the mitochondrial control region of Fringilline Finches (*Fringilla* spp.) and the Greenfinch (*Carduelis chloris*). Molecular Biology and Evolution 14: 173-184.

MARSHALL, H. D. & BAKER, A. J. 1999. Colonisation history of atlantic island Common Chaffinches (*Fringilla coelebs*) revealed by mitochondrial DNA. Molecular Phylogenetics and Evolution 11: 201-222.

MATHEWS, D.H., BURKARD, M. E. TURNER, D. H. 1999. RNAstructure: a computer program for Windows 95/NT available at http://rna.chem.rochester.edu.

MAY, R. M., ENDLER, J. A. & McMURTRIE, R. E. 1975. Gene frequency clines in the presence of selection opposed by gene flow. American Naturalist 109: 659-676.

MAYR, E. 1942. Systematics and the Origin of Species. Columbia University Press,

New York.

MAYR, E. 1963. Animal Species and Evolution. Harvard University Press. Cambridge.

MAYR, E. 1970. Populations, Species and Evolution. Belknap Press, Cambridge, Massachusetts.

MCLENNAN, D. A., BROOKS, D. R. & MCPHAIL, J. D. 1988. The benefits of communication between comparative ethology and phylogenetic systematics: a case study using gasterosteid fishes. Canadian Journal of Zoology 66: 2177-2190.

MINDELL, D. P., SORENSON, M. D. & DIMCHEFF, D. E. 1998. Multiple independent origins of mitochondrial gene order in birds. Proceedings of the National Academy of Sciences, USA 95: 10693-10697.

MINDELL, D. P., SORENSON, M. D., HUDDLESTON, C. J., MIRANDA, H. C., KNIGHT, A., SAWCHUK, S. J. & YURI, T. 1997. Phylogenetic relationships among and within select avian orders based on mitochondrial DNA. In D. P. Mindell (ed) Avian Molecular Evolution and Systematics. Academic Press, San Diego.

MOORE, W. S. & DEFILIPPIS, V. R. 1997. The window of taxanomic resolution for phylogenies based on mitochondrial cytochrome b. In D. P. Mindell (ed) Avian Molecular Evolution and Systematics. Academic Press, San Diego.

MOORE, W. S. & KOENIG, W. D. 1986. Comparative reproductive success of yellow-shafted, red-shafted and hybrid flickers across a hybrid zone. Auk 103: 42-51.

MOORE, W. S. 1987. Random mating in the Northern Flicker hybrid zone: implications for the evolution of bright and contrasting plumage patterns in birds. Evolution 41: 539-546.

MUNDINGER, P. C. 1982. Microgeographic and macrogeographic variation in the

aquired vocalisations of birds. In D. E. Kroodsma & E. H. Miller (eds) Acoustic Communication in Birds volume 2: 147-208. Academic Press, New York.

NEI, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

NELSON, G. 1989. Species and Taxa: Systematics and Evolution. D. Otte & J. A. Endler (eds) Speciation and Its Consequences: 60-81. Sinauer Associates. Sunderland, Massachusetts.

PAGE, L. M. & JOHNSTON, C. E. 1990. The breeding behavior of *Opsopoeodus emiliae* (Cyprinidae) and its phylogenetic implications. Copeia 4: 1176-1180.

PARSONS, T. J., OLSON, S. L. & BRAUN, M. J. 1993. Unidirectional spread of secondary sexual plumage traits across an avian hybrid zone. Science 260: 1643-1646.

PATERSON, A. M., WALLIS, G. P. & GRAY, R. D. 1995. Penguins, petrels and parsimony: does cladistic analysis of behaviour reflect seabird phylogeny. Evolution 49: 974-989.

PATERSON, H. E. H. 1985. The Recognition Concept of species. In E. S. Vrba (ed) Species and Speciation: 21-30. Transvaal Museum Monograph No. 4. Pretoria.

PAYNE, R. B. 1986. Bird songs and avian systematics. In R. F. Johnston (ed) Current Ornithology Volume 3: 87-125. Plenum Press. New York.

PIERTNEY, S. B., MACCOLL, A. D. C., BACON, P. J., RACEY, P. A., LAMBIN, X. & DALLAS, J. F. 2000. Matrilineal genetic structure and female-mediated gene flow in Red Grouse (*Lagopus lagopus scoticus*): an analysis using mitochondrial DNA. Evolution 54: 279-289.

PRUM, R. O. 1990. Phylogenetic analysis of the evolution of display behavior in the

Neotropical Manakins (Aves: Pipridae). Ethology 84: 202-231.

QUIESTIAU, S. GIELLY, L., CLOUET, M. & TABERLET, P. 1999. Phylogeographical evidence of gene flow among Common Crossbill (*Loxia curvirostra*, Aves, Fringillidae) populations at the continental level. Heredity 83: 196-205.

QUINN, T. W. & WILSON, A. C. 1993. Sequence evolution in and around the mitochondrial control region in birds. Journal of Molecular Evolution 37: 417-425.

QUINN, T. W. 1997. Molecular evolution of the mitochondrial genome. In D. P. Mindell (ed) Avian Molecular Evolution and Systematics. Academic Press, San Diego.

RAMIREZ, V., SAVOIE, P. & MORAIS, R. 1993. Molecular characterization and evolution of a duck mitochondrial genome. Journal of Molecular Evolution 37: 296-310.

RAND, D. M. & HARRISON, R. G. 1989. Ecological genetics of a mosaic hybrid zone: mitochondrial, nuclear and reproductive differentiation of crickets by soil type. Evolution 43: 432-449.

RANDI, E. & LUCCHINI, V. 1998. Organization and evolution of the mitochondrial DNA control region in the avian Genus *Alectoris*. Journal of Molecular Evolution 47: 449-462.

RIEKERT, B. R. & CLINNING, C. F. 1985. The use of artificial nest boxes in the Daan Viljoen Game Park. Bokmakierie 37: 84-86.

ROHWER, S. & WOOD, C. 1998. Three hybrid zones between Hermit and Townsend's Warblers in Washington and Oregon. The Auk 115: 284-310.

SACCONE, C., PESOLE, G. & SBISÁ, E. 1991. The main regulatory region of

mammalian mitochondrial DNA: Structure-Function model and evolutionary pattern. Journal of Molecular Evolution 33: 83-91.

SAITOU, N. & NEI, M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425.

SANFT, K. 1960. Bucerotidae (Aves/Upupae). Das Tierreich 76: 1-176.

SANTALUCIA, Jr., J. 1998. A unified view of polymer, dumbell and oligonucleotide DNA nearest-neighbour thermodynamics. Proceedings of the National Academy of Sciences, USA 95: 1460-1465.

SATO, A., O'HUIGIN, C., FIGUEROA, F., GRANT, P. R., GRANT, B. R., TICHY, H. & KLEIN, J. 1999. Phylogeny of Darwin's finches as revealed by mtDNA sequences. Proceedings of the National Academy of Sciences, USA 96: 5101-5106.

SAUNDERS, M. A. & EDWARDS, S. V. 2000. Dynamics and phylogenetic implications of mtDNA control region sequences in New World Jays (Aves: Corvidae). Journal of Molecular Evolution 51: 97-109.

SBISÁ, E., TANZARIELLO, F., REYERS, A, PESOLE, G. & SACCONE, C. 1997. Mammalian mitochondrial D-loop region structural analysis: identification of new conserved sequences and their functional and evolutionary implications. Gene 205: 125-140.

SHIELDS, G. F. & HELM-BYCHOWSKI, K. M. 1988. Mitochondrial DNA of birds. Current Ornithology 5: 273-294.

SMUTS, J. C. 1926. Holism and Evolution. N & S Press, Cape Town.

SOKAL, R.R. 1974. The species problem reconsidered. Systematic Zoology 22:360-374.

SORENSON, M. D. & FLEISCHER, R. C. 1996. Multiple independent transpositions of mitochondrial DNA control region sequences to the nucleus. Proceedings of the National Academy of Sciences, USA 93: 15239 - 15243.

SOUTHERN, S. O., SOUTHERN, P. J. & DIZON, A. E. 1988. Molecular characterisation of a cloned dolphin mitochondrial genome. Journal of Molecular Evolution 28: 32-42.

SPERLING, F. A. H. & SPENCE, J. R. 1991. Structure of an asymmetric hybrid zone between two water strider species (Hemiptera: Gerridae: *Limnoporus*). Evolution 45: 1370-1383.

SULLIVAN, B. K. & MALMOS, K. B. 1994. Call variation in the Colorado River Toad (*Bufo alvarius*): behavioral and phylogenetic implications. Herpetologica 50: 146-156.

SWOFFORD, D. L. 1999. Phylogenetic Analysis Using Parsimony (PAUP), version 4.03b3. Illinois Natural History Survey, Champaign.

TEMPLETON, A. R. 1989. The meaning of species and speciation: A genetic perspective. In D. Otte & J. A. Endler (eds) Speciation and Its Consequences. Sinauer, Sunderland, Massachusetts.

TEMPLETON, A. R. 1998. Species and speciation: geography, population structure, ecology, and gene trees. In D. J. Howard & S. H. Berlocher (eds) Endless Forms: Species and Speciation. Oxford University Press, New York.

THIELCKE, G. 1969. Geographic variation in bird vocalizations. In R. A. Hinde (ed) Bird Vocalizations: their relations to current problems in biology and psychology: 311-339. Cambridge University Press, Cambridge.

TRACY, T. T. & BAKER, M. C. 1999. Geographic variation in syllables of House Finch songs. Auk, 116: 666-676.

TRÉCA, B. & ERARD, C. 2000. A new subspecies of the Red-billed Hornbill *Tockus erythrorhynchus* from West Africa. Ostrich 71: 363-366.

VAN DER MERWE, J. H. 1983. National Atlas of South West Africa (Namibia). Directorate Development Co-ordination. Windhoek, Namibia.

WENINK, P. W., BAKER, A. J. & TILANUS, M. G. J. 1994. Mitochondrial control-region sequences in two shorebird species, the Turnstone and the Dunlin, and their utility in population genetic studies. Molecular Biology and Evolution 11: 22-31.

ZHANG, D. X. & HEWITT, G. M. 1996. Nuclear integrations: challenges for mitochondrial DNA markers. TREE 11: 247-251.

ZINK, R. M., WELLER, S. J. & BLACKWELL. 1998. Molecular phylogenetics of the Avian genus *Pipilo* and a biogeographic argument for taxonomic uncertainty. Molecular Phylogenetics and Evolution 10: 191-201.

# Appendices

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MINISTRY OF ENVIRONMENT AND TOURISM

Division: Specialist Support Services
Private Bag 13306
Windhoek, NAMIBIA
Enq: Chief Warden: Permit Office

Tel: +264-61-263131
Fax: +264-61-259101

| | |
|---|---|
| PERMIT NO: | 29674 P |
| VALID FROM: | 01.10.98 |
| VALID TO: | 30.09.99 |

# GENERAL PERMIT

Name: W. Delport

Residential address:

Postal address: University of Pretoria

Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975)

To do behavioural studies on Redbilled Hornbills and collect blood samples for genetic analysis in the Otavi Mountain region. Videotape and sound record Redbilled Hornbills in Etosha National Park, Waterberg Plateau Park and Daan Viljoen Game Reserve.

Remarks/conditions:

Subject to attached list of conditions.

MINISTRY OF ENVIRONMENT AND TOURISM
Official Act / Gate Stamp
REPUBLIC OF NAMIBIA
22 SEP 1998
WINDHOEK
PRIVATE BAG 13306 WINDHOEK
Tel. 061-63131 Fax 061-63195

Authorizing officer

IMPORTANT

This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder and the holder is subject to all such conditions and regulations.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

| MINISTRY OF ENVIRONMENT AND TOURISM | PERMIT NO: | 32756 |
|---|---|---|
| Division: Specialist Support Services<br>Private Bag 13306<br>Windhoek, NAMIBIA<br>Enq.: Chief Warden: Permit Office<br>Tel: +264-61-263131<br>Fax: +264-61-259101 | VALID FROM: | 01-10-99 |
| | VALID TO: | 31-09-2000 |

# GENERAL PERMIT

Name: Wayne Pelport

Residential address: University of Pretoria

Postal address: RSA

Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975)

To do behavioural studies on Redbilled Hornbills and collect blood samples for genetic analysis in the Otavi Mountain region. Videotape and sound record Redbilled Hornbills in the Etosha National Park, Waterberg Plateau Park and Daan Viljoen Game Reserve.

Remarks / Conditions:

Subject to attached list of Conditions.

IMPORTANT: This permit is not valid if altered in any way

Official MET date stamp

Authorizing officer

**IMPROTANT**

This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all such conditions and regulations

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MINISTRY OF ENVIRONMENT AND TOURISM
Division Special Support Services
Private Bag 13306
Windhoek, NAMIBIA
Enq: Chief Warden Permit Office
Tel: +264-61-253131
Fax: +264-61-259101

| | |
|---|---|
| PERMIT NO: | 33057 P |
| VALID FROM: | 01 · 11 · 1999 |
| VALID TO: | 30 · 10 · 2000 |

# GENERAL PERMIT

Name: Wayne Delport

Residential address: University of Pretoria

Postal address: RSA

Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975)

To Shoot & Collect not more than thirty (30) Redbilled Hornbills in Northern Namibia, excluding protected areas

Remarks / Conditions:

Subject to attached List of Conditions

IMPORTANT: This permit is not valid if altered in any way

G C Craig
Authorizing officer

MINISTRY OF ENVIRONMENT AND TOURISM
Official Receipt
REPUBLIC OF NAMIBIA
05 NOV 1999
WINDHOEK
PRIVATE BAG 13306 WINDHOEK
Tel 061-63131 Fax 061-259101

IMPORTANT:
This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all such conditions and regulations



This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all such conditions and regulations