BEYOND DNA SEQUENCING: INTEGRATIVE APPROACHES TO RESOLVING SELECTED HIGHER AND LOWER TAXONOMIC PROBLEMS IN AFROTROPICAL CHIROPTERA

LEIGH ROSANNE RICHARDS

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Supervisory Committee:

Prof. Jennifer M. Lamb

Prof. Peter J. Taylor

Dr M. Corrie Schoeman

Dr Steven M. Goodman

DEDICATION

Dedicated to the memories of grandpa Barker and grandpa Richards

"*It seems to me that the natural world is the greatest source of excitement; the greatest source of visual beauty; the greatest source of intellectual interest. It is the greatest source of so much in life that makes life worth living*."

Sir David Attenborough

ABSTRACT

Of the approximate 300 currently recognised bat species known from the Afrotropics, very few have been studied in sufficient detail to a) provide accurate species and distributional limits for extant taxa, b) identify possible cryptic species, and c) ascertain the closest sister lineage of numerous taxonomic groups. For those species where DNA-based phylogenies are available, the use of additional taxonomic markers and methods has provided further insights into the evolutionary history of certain extant chiropteran groups. This work comprises a series of systematic studies of African and Malagasy Chiroptera aimed at investigating sequence-based evolutionary hypotheses of higher and lower level taxa using comparative molecular cytogenetic and morphometric techniques.

Efforts were directed at resolving taxonomic inconsistencies of chiropteran taxa from the African subregion and/or Madagascar, for which there is a general paucity of comprehensive and/or resolved phylogenies. Taxa belonging to the families Pteropodidae, Hipposideridae, Myzopodidae, and Molossidae were chosen for study because molecular-based have failed to provide consensus regarding evolutionary relationships amongst the above-mentioned taxonomic groups, or are in stark contrast to phylogenies based on morphological data. In addition, molecular cytogenetics and geometric morphometric approaches were used because they have had been applied in few evolutionary studies of Afrotropical bats.

With the exception of a few karyotypic descriptions, scant data are available that details the chromosomal diversity and karyotypic evolution of bats from Madagascar in relation to their conspecifics or congenerics on other continents. To understand better the mechanisms that may have structured the karyotypes of extant Malagasy Chiroptera and the utility of chromosomal characters in retracing their evolutionary history, eight species from seven families were analysed using G- and C-banding and chromosome painting. Robertsonian (Rb) fusions and fissions were the dominant mode of genome restructuring amongst taxa and, for the most part, proved useful characters for investigations of phylogenomic relationships amongst families and genera.

Chromosomal data generated from painting studies employing *Myotis myotis* (MMY) chromosomal probes, produced phylogenetically important characters that supported two conflicting hypotheses regarding the evolutionary affinities of the Myzopodidae, a family of bats endemic to Madagascar. The Rb fusion MMY 9+11 detected in Myzopodidae, also common to Phyllostomidae, could suggest a close association of *Myzopoda aurita* with the superfamily Noctilionoidea. However, the Rb fusion MMY 3+4 that is also present in vesper bats, suggests closer evolutionary ties between *M. aurita* and the Vespertilionoidea. A sex-autosome translocation, a cytogenetic character previously confined to phyllostomid and vespertilionid

bats, was also detected in *M. aurita* casting further uncertainties on the evolutionary origins of this deep-branching species. This study highlighted the need for more refined cytogenetic investigations based on human-derived chromosomal paints and the application of high-resolution bacterial artificial chromosomal (BACs) probes to map intrachromosomal breakpoints and/or subchromosomal rearrangements in the genome of *Myzopoda*.

Heterochromatic polymorphisms and inversions appear to be important mechanisms of karyotypic evolution amongst pteropodid genera. Painting data revealed that at least five structural arrangements might be linked to the evolutionary divergence of pteropodine and rousettine fruit bats. A cryptic pericentric inversion was detected in the genome of *Pteropus rufus* corresponding to the homologue of MMY 4+19 (equivalent to HSA3+21); an ancestral syntenic character proposed for eutherian mammals. Proposed synapomorphies of the rousettine clade, as defined by molecular DNA studies, include the derived state of the MMY 4+19 homologue and the non-centric fusion of MMY 16/17+24 homologue.

Integration of painting data on *Hipposideros commersoni* with published comparative maps of other hipposiderids enabled a brief revision of the postulated ancestral hipposiderid chromosomal complement. These data disputed previously proposed chromosomal synapomorphies of Hipposideridae and supported the basal position of *H. commersoni* within the genus. The inclusion of other hipposiderid genera, in particular Malagasy *Paratriaenops* and southern African *Cloeotis*, in chromosome painting studies may allow for further inferences regarding the evolutionary history of this diverse family.

Morphometric approaches were employed to resolve uncertainties concerning species-level relationships within Afrotropical *Otomops*. Multivariate analyses delineated three well-supported morphological groups that corresponded to recently described genetic lineages and revealed several species-specific morphological traits for taxonomic diagnoses. *Otomops* from Djibouti, Ethiopia, Kenya, and Yemen constitute an undescribed morphologically and genetically cohesive group that requires a formal taxonomic description. Understanding the ecological and possible physiological adaptive value of morphological variation can provide valuable insights into the evolutionary history of this Afrotropical species complex.

This work has provided further insights into the systematics of certain Afrotropical Chiroptera through the use of molecular cytogenetic and geometric morphometric techniques. Specifically, it has facilitated the interpretation of ancestral, independent and convergent chromosomal characters in the evolution of Afrotropical taxa belonging to the families Pteropodidae, Hipposideridae, and Myzopodidae, and has also elucidated lineage-specific morphological attributes in members of the genus *Otomops* thereby advancing our understanding of chiropteran diversity within the region.

PREFACE

The experimental work described in this thesis was carried out in the School of Life Sciences, University of KwaZulu-Natal (Westville Campus) and Department of Botany and Zoology, Evolutionary Genomics Group (EGG) Laboratory, University of Stellenbosch. Specimens were collected under permits issued by the Ministry of Environment, Water, Forests, and Tourism (Madagascar) and Ezemvelo KZN Wildlife (South Africa). Since October 2010 to August 2013, I have completed fieldwork, laboratory-based analyses and writing whilst being in the full-time employment of the eThekwini Municipality as the Curator of Mammals at the Durban Natural Science Museum.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

L. R. RICHARDS

DECLARATION 1 – PLAGIARISM

I, **Leigh Rosanne Richards** declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted:

   4.1. Their words have been rewritten but the general information attributed to them has been referenced.

   4.2. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References section.

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DECLARATION 2 – PUBLICATIONS

This thesis has been prepared according to the format of manuscripts for publication in peer-reviewed journals. This has resulted in variation in the format of the three research chapters and some repetitive text particularly in the case of the research method sections. The contribution of PhD candidate Leigh Rosanne Richards and co-authors to scientific papers presented in the thesis are listed below:

Publication 1

Richards LR, Rambau RV, Lamb JM, Taylor PJ, Yang F, Schoeman MC, Goodman SM (2010) Cross-species chromosome painting in bats from Madagascar: the contribution of Myzopodidae to revealing ancestral syntenies in Chiroptera. *Chromosome Research* **18:** 635–653.

LR Richards conceived the original idea of the manuscript, participated in field sampling of bats in Madagascar, was responsible for cell culture, conducted the analyses and wrote the first and final versions of the published manuscript. RV Rambau supervised with cell culture and contributed to the final version of the manuscript. SM Goodman was responsible for coordinating field sampling of bats in Madagascar, securing export permits, and contributed to the final version of the manuscript. PJ Taylor and MC Schoeman assisted with field sampling and contributed to the final version of the manuscript. F Yang provided the *Myotis myotis* chromosomal probes and contributed to the final version of the manuscript. JM Lamb provided funding towards operational costs, subsistence and travel costs, and contributed to the final version of the manuscript.

Publication 2

Richards LR, Rambau RV, Goodman SM, Taylor PJ, Schoeman MC, Yang F, Lamb JM (unsubmitted manuscript) Karyotypic evolution in Malagasy flying foxes (Pteropodidae, Chiroptera) and their rhinolophoid relatives as determined by comparative chromosome painting (*formatted according to the guidelines of Cytogenetics and Genome Research*).

LR Richards conceived the original idea of the manuscript, participated in field sampling of bats in Madagascar and South Africa, was responsible for cell culture, conducted the analyses, and wrote the first and final versions of the prepared manuscript. RV Rambau supervised with cell culture and contributed to the final version of the manuscript. SM Goodman was responsible for coordinating field sampling of bats in Madagascar, securing export permits, and contributed to the final version of the manuscript. PJ Taylor and MC Schoeman assisted with field sampling and contributed to the final version of the manuscript. F Yang provided the *Myotis myotis* chromosomal probes. JM Lamb provided funding towards operational costs, subsistence and travel costs, and contributed to the final version of the manuscript.

Publication 3

Richards LR, Taylor PJ, Schoeman MC, Goodman SM, Van Daele PAAG, Lamb JM (2012) Cranial size and shape variation in Afrotropical *Otomops* (Mammalia: Chiroptera: Molossidae): testing species limits using a morphometric approach. *Biological Journal of the Linnean Society* **106:** 910–925.

LR Richards co-conceived the original idea of the manuscript, was partly responsible for obtaining loaned material, undertook all data collection and the analysis thereof, and wrote the first and final versions of the published manuscript. PJ Taylor co-conceived the original idea of the manuscript, was partly responsible for obtaining loaned material, and contributed to the final version. MC Schoeman assisted with data analysis and contributed to the final version of the manuscript. SM Goodman was responsible for collection of specimens in Madagascar and contributed to the final version of the manuscript. PAAG Van Daele provided valuable samples and contributed to the final version of the manuscript. JM Lamb provided funding towards operational costs, subsistence and travel costs, and contributed to the final version of the manuscript.

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To my dearest friend and colleague Taryn Ralph, thank you for your friendship and support throughout the past twelve years. I would like to thank especially Theshnie Naidoo for assisting with ordering all my cytogenetic reagents and media. Dr Paula Sommer and her post-graduate students are thanked for allowing me access to their cell culture facilities at the School of Life Sciences.

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CHAPTER ONE

GENERAL INTRODUCTION

AFROTROPICAL CHIROPTERA

Bats (Order Chiroptera) belong to the second most speciose group of placental mammals. With approximately 1260 recorded species, they account for at least 23% of the 5500 described mammalian species (Fenton 2012; Wilson & Reeder 2005). Currently there are 20 recognised families of extant bats: Cistugonidae, Craseonycteridae, Emballonuridae, Furipteridae, Hipposideridae, Megadermatidae, Miniopteridae, Molossidae, Mormoopidae, Mystacinidae, Myzopodidae, Natalidae, Noctilionidae, Nycteridae, Phyllostomidae, Pteropodidae, Rhinolophidae, Rhinopomatidae, Thyropteridae and Vespertilionidae (Simmons 2005; Miller-Butterworth *et al*. 2007; Lack *et al*. 2010). The unique capability of powered flight amongst bats has allowed them to colonise most regions of the world, apart from the Arctic, Antarctica and several isolated oceanic islands (Mickleburgh *et al*. 2002).

The greatest bat biodiversity is concentrated within the tropics. The Afrotropical region, in the biogeographical sense, encompasses sub-Saharan Africa, the southwestern fringes of the Arabian Peninsula (African subregion), and Madagascar and its neighbouring oceanic islands (Malagasy subregion) (Udvardy *et al*. 1975; Olson *et al*. 2001; Fig 1). The region boasts approximately 300 chiropteran species belonging to 12 families and 56 genera (ACR 2012; Goodman *et al*. 2011, 2012a,b). Two families (Cistugonidae and Myzopodidae) and 28 genera are endemic to the region. The highest species diversity for bats and other small mammals is concentrated within the eastern regions of southern Africa (Schoeman *et al*. in press), the West African forests, Eastern Arc forests, East African coastal forests and Madagascar (Myers *et al*. 2000; Ceballos & Ehrlich 2006). Madagascar is of particular biogeographical interest and conservation significance due to the islands unique biota and extraordinarily high levels of endemism (Goodman & Benstead 2005). Relative to other mammalian taxa inhabiting the island, the bat fauna is the least understood and studied.

Very few widespread bat species have been studied in detail across portions of their range within the Afrotropics, and, hence, little information is available on their biology and ecology. Incomplete biological inventories of certain areas, insufficient specimen material to support taxonomic studies and the poor resolution of many cryptic species complexes has led to a gross underestimation of the true number of bat species occurring in the region. At least 10% of all extant Afrotropical Chiroptera are threatened (Critically Endangered, Endangered or Vulnerable), with a further 5% listed as Near Threatened (IUCN 2013). Approximately 55 taxa are listed as Data Deficient due to limited information available to formulate measures of their conservation status. An additional 30 taxa of questionable taxonomic status or more recently described species have yet to be evaluated.

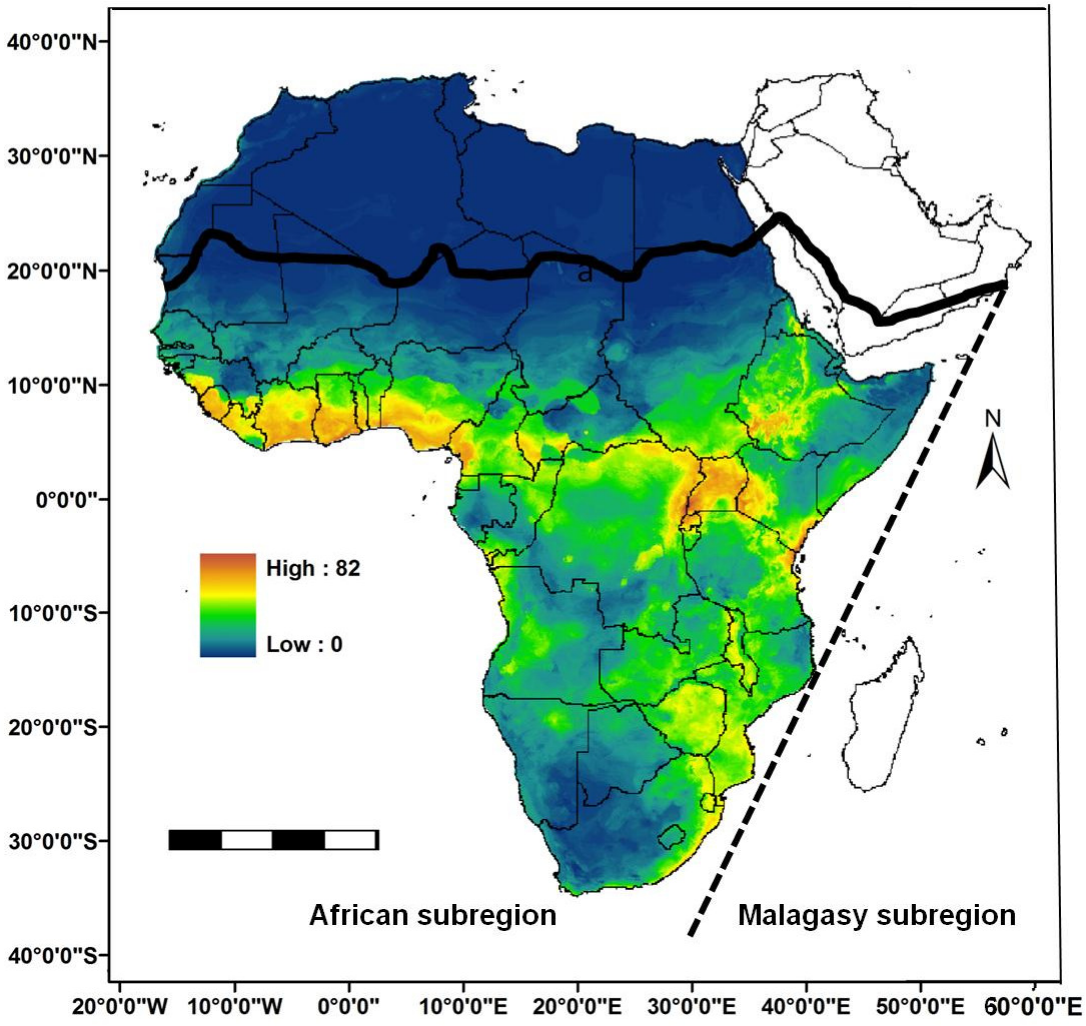

Figure 1. The Afrotropical region showing the demarcation of the African and Malagasy subregions. The black solid line indicates the separation between the Afrotropical and Palaearctic regions. The coloured areas depict predicted bat biodiversity throughout Africa as modelled using Maxent (Monadjem, Schoeman, and Smith unpublished data).

RECENT ADVANCES IN SYSTEMATIC STUDIES OF AFROTROPICAL CHIROPTERA

The new age of discovery

Within recent decades, concerted efforts have been made to improve our understanding of the diversity, taxonomy, distribution and natural history of bats worldwide. Countries within the Afrotropics, in particular Madagascar, have benefited from this renewed attention, with national and international research enriching our knowledge of the bat fauna within the region (Hoffman *et al*. 2009). With this collective effort have come rediscoveries of rare species (e.g. Monadjem *et al*. 2005) and the discovery of many new species (reviewed in Ceballos & Ehrlich 2009;

Hoffman *et al*. 2009; Monadjem *et al*. 2010). Inventories of previously poorly surveyed areas and/or the new collections from remote areas have yielded morphologically distinct species belonging to several families, previously not known to science (e.g. *Myzopoda schliemanni*, Goodman, Rakotondraparany, Kofoky 2007; *Pipistrellus raceyi*, Bates, Ratrimomanarivo, Harrison, Goodman 2006; *Rhinolophus maendeleo*, Kock, Csorba, Howell 2000; *R. sakejiensis*, Cotterill 2002; *R. ziama*, Fahr Vierhaus, Hutterer, Kock 2002; *Scotophilus tandrefana*, Goodman, Jenkins, Ratrimomanarivo 2005).

From 1988 until present, approximately 44 new bat species have been described from Africa and the Malagasy subregion (Hoffman *et al*. 2009; ACR 2012; Taylor *et al*. 2012). In Madagascar alone, there has been an increase from an estimated 30 species (Eger & Mitchell 1996, 2003), to over 44 recorded species (Goodman 2011; Goodman *et al*. 2011, 2012b). Approximately 80% of all Malagasy bat species are endemic, whilst those non-endemics are shared with neighbouring oceanic islands or mainland Africa (Goodman 2011; Goodman *et al*. 2012b). Most of these new species discoveries have originated from the compilation of new specimen material that disputed traditional classifications and thus warranted systematic revisions of certain taxonomic groups (Yoder *et al*. 2005). This refinement of species boundaries and definition of their historical diversification has been bolstered by an increase in the size of available museum collections. Specimen collections provide an invaluable resource for taxonomists and systematists in understanding biodiversity, both past and present. Traditionally geared towards the preservation and comparison of organismal phenotypes, which was the primary means of species recognition, biological repositories now play a significant role in enhancing both biodiversity science and genomic studies (Hanner & Gregory 2007).

The molecular phylogenetic revolution

DNA sequence data and molecular phylogenetics have transformed our perception of the evolutionary relationships amongst Chiroptera. The ease, precision and efficiency of DNA sequencing methods, facilitated by polymerase chain reaction (PCR) based techniques, have allowed for the rapid study of a greater number of taxa than was possible in previous years and have entrenched the use of sequence data as a preferred means for phylogenetic reconstruction and inference (Galtier *et al*. 2009; Winker 2009; McCormack *et al*. 2013). Where traditional comparative morphological studies have failed to provide consensus, molecular DNA approaches have, for the most part, provided robust phylogenies that have resolved several contentious hypotheses concerning evolutionary associations at both higher and lower taxonomic levels.

Higher-level systematic studies of the Chiroptera were traditionally dominated by morphological data that supported the existence of two reciprocally monophyletic clades: the Megachiroptera (largely non-echolocating bats belonging to the family Pteropodidae) and the Microchiroptera (echolocating bats) (Koopman 1994a; Simmons 1998; Simmons & Geisler

1998). Molecular studies have revolutionised this long-established classification of the Chiroptera by revealing the paraphyly of Microchiroptera and positioning rhinolophoid microbats as the sister-taxon to the pteropodids to the exclusion of other previously recognised microbat families (Van Den Bussche & Hoofer 2004; Eick *et al*. 2005; Teeling *et al*. 2005; Fig 2). Currently two subordinal and four superfamilial groups are recognized: Yangochiroptera or Vespertilioniformes consisting of the Emballonuroidea, Noctilionoidea and the Vespertilionoidea, and the Yinpterochiroptera or Pteropodiformes, which comprises the Pteropodidae and several families belonging to the Rhinolophoidea (Craseonycteridae, Rhinopomatidae, Megadermatidae, Hipposideridae, and Rhinolophidae) (Eick *et al*. 2005; Teeling *et al*. 2005).

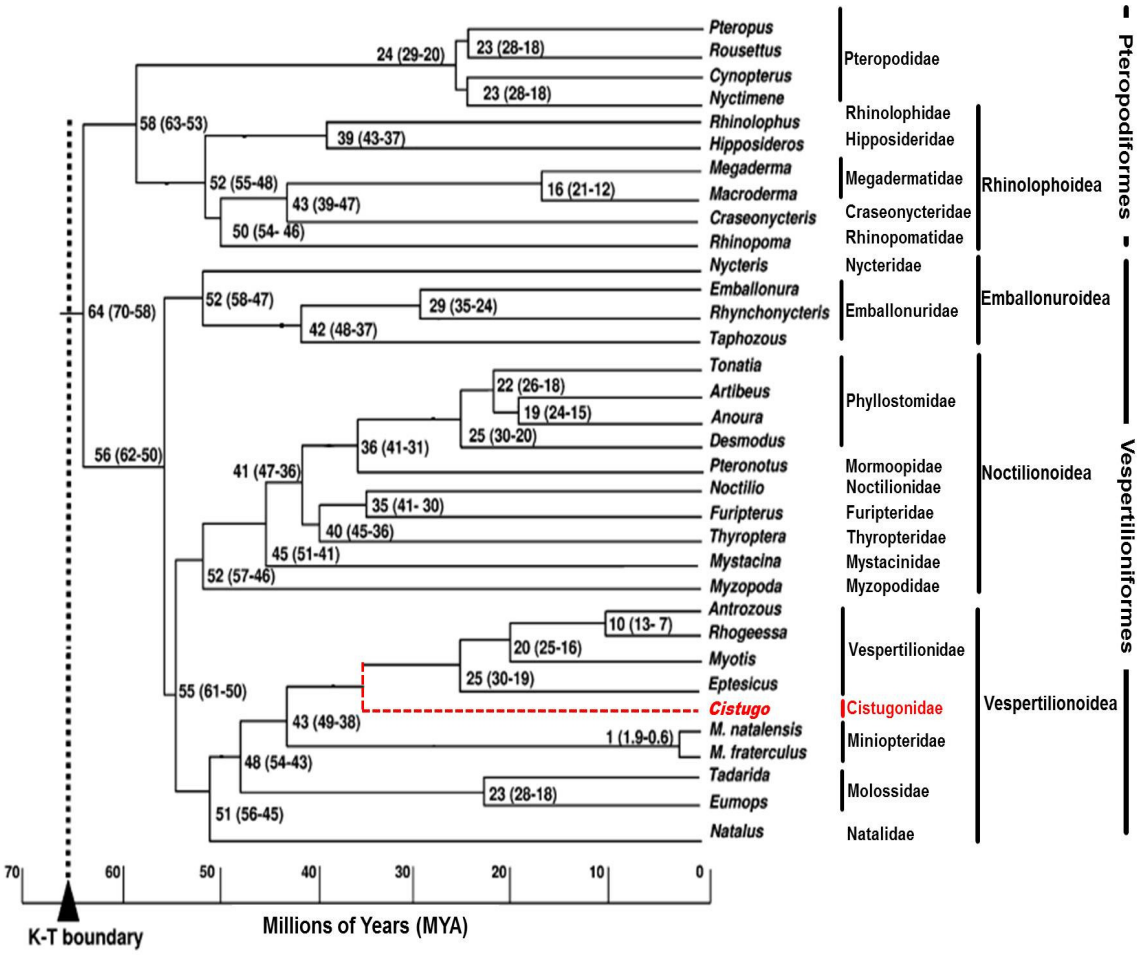

Figure 2. Molecular consensus tree and timescale derived from Miller-Butterworth *et al*. (2007) that portrays the current understanding of phylogenetic relationships amongst Chiroptera at the familial, superfamilial and subordinal levels. Nodal numbers indicate molecular dates in millions of years with 95% credibility intervals indicated in parentheses. The position of Cistugonidae is based on the phylogeny of Lack *et al*. (2010).

Further genetic studies have led to the discoveries of novel families previously classified within Vespertilionidae. For instance, traditional classifications have long recognised *Miniopterus* as the sole genus of the vespertilionid subfamily Miniopterinae. Recent analyses based on mitochondrial DNA (mtDNA) revealed the basal positioning and genetic distinctiveness of this group from all other vespertilionid species (Hoofer & Van Den Bussche 2003; Van Den Bussche & Hoofer 2004). Analyses based on mtDNA and nuclear markers provided conclusive evidence and consensus for the recognition of Miniopteridae (Eick *et al*. 2005; Miller-Butterworth *et al*. 2007). Similarly, Stadelmann *et al*. (2004) used cytochrome *b* (cyt b) data to provide strong support for the removal of *Cistugo leuseri* and *C*. *seabrae* from the *Myotis* genus and the recognition of *Cistugo* as a separate and distinct subfamily of Vespertilionidae. Using a concatenated data set of one mitochondrial and six nuclear genes, Lack *et al*. (2010) provided definitive evidence for establishing *Cistugo* as a hitherto unrecognised and endemic African bat family, designated Cistugidae and later reclassified to Cistugonidae (Van Cakenberghe & Seamark 2011).

The integrative consensus – beyond DNA sequencing and molecular phylogenies

Despite the major advances to resolve the branches of the chiropteran phylogenetic tree using nucleotide sequence data, some uncertainty nevertheless surrounds the evolutionary arrangement of certain families and the positioning of several genera and species within particular lineages. Ambiguities may arise from the lack of species-level phylogenies for numerous bat lineages that are needed to fully resolve higher-level relationships within Chiroptera. Incongruities between molecular DNA reconstructions resulting from differences in taxon sampling (Rokas & Carroll 2005), disparities in the number and/or type of gene loci utilised (Eick *et al*. 2005; Galtier *et al*. 2009; Vallo *et al*. 2012), and the improper choice of out-group (Van Den Bussche & Hoofer 2004), can contribute towards misinterpretations of the phylogenetic relationships within Chiroptera. Incomplete lineage sorting and/or introgression between sister taxa (e.g. Baird *et al*. 2008) may also hamper phylogenetic inferences. Furthermore genetic variation is neutral or near neutral and is the result of random processes that do not drive evolutionary divergence in the process of speciation (Winker 2009). Hence, the use of genetic data without considering other characters or traits subject to selective pressures may present a unidimensional view of complex evolutionary processes (Winker 2009).

Accurate evolutionary reconstruction and species delimitation relies increasingly on the combined analysis and/or accumulation of evidence from multiple types of taxonomic characters. Taxonomic congruence between studies based on autonomous data sets provides strong evidence that latent historical patterns have been uncovered and can maximise the resolution of evolutionary lineages (Hillis 1987). A diverse array of taxonomic markers and methods can be used as independent means of assessing the degree of support for various genetic clades and/or to better understand those evolutionary processes that have led to

observed DNA sequence divergences (Simmons 2000; Wetterer *et al*. 2000), which include palaeontological data, morphometric characters, karyotypic data, bioacoustic information, and ontogenetic data, to name but a few. This multi-disciplinary method, collectively termed integrative taxonomy or integrative biology, is fast becoming a widely accepted discipline in modern systematics (Dayrat 2005; Padial *et al*. 2010).

Within the recent past, there has been an increase in systematic studies of Afrotropical Chiroptera that have adopted an integrative approach to provide a more complete and insightful interpretation of species diversity and other complex phenomena that underlie speciation within the Rhinolophidae (Stoffberg *et al*. 2012; Taylor *et al*. 2012; Benda & Vallo 2012), Hipposideridae (Benda & Vallo 2009; Vallo *et al*. 2008, 2011a), Emballonuridae (Goodman *et al*. 2012b), Molossidae (Ratrimomanarivo *et al*. 2007, 2009; Goodman *et al*. 2010a), Miniopteridae (Miller-Butterworth *et al*. 2005; Goodman *et al*. 2009a,b; Ramasindrazana *et al*. 2011) and Vespertilionidae (Vallo *et al*. 2011b, 2012; Goodman *et al*. 2012a; Monadjem *et al*. 2013). In most cases, sequence data have provided evolutionary hypotheses allowing for the *a posteriori* categorization of taxa that have facilitated the discovery of apomorphic attributes supporting genetic clades (Ramasindrazana *et al*. 2011). It has also proved valuable in determining the nature of important adaptations, and whether they have an independent or convergent origin (Teeling *et al*. 2002). Hence, molecular DNA phylogenies can be the first step in providing an evolutionary context for the advancement of bat systematic studies.

ALTERNATIVE TAXONOMIC CHARACTERS AND METHODS

Molecular cytogenetic and geometric morphometric data, whilst becoming increasingly recognised as valuable tools in mammalian systematic studies, have been relatively under-utilised as methods that can support molecular studies of Afrotropical bats. Below is a brief review of the two methods as they apply to taxonomic studies of Chiroptera and other small mammals.

The molecular cytogenetic approach

Chromosomes are regarded as heritable independent units of the nuclear genome that can carry mutations and are thus considered important evolutionary characters (Dobigny *et al*. 2004a). Structural chromosomal rearrangements, such as inversions and translocations, can represent large-scale genomic mutational changes occurring within particular lineages that are the drivers of karyotypic evolution (Rokas & Holland 2000; Murphy *et al*. 2004) and, in certain instances, speciation (Rieseberg 2001; Navarro & Barton 2003). Chromosomal speciation may promulgate when hybrid fertility (or viability) is reduced because of malsegregation or mispairing

of homologous chromosome during meiosis (White 1978). This chromosomal mispairing may result from structural chromosome changes produced for example, by reciprocal translocations, tandem fusions, monobrachial fusion events, inversions, and X-autosome translocations (White 1973; Baker & Bickham 1986). Karyotypic evolution advances at a slower pace than nucleotide evolution (Murphy *et al*. 2004), thus chromosomal rearrangements are considered rare genomic changes (RCGs *sensu* Rokas & Holland 2000) capable of providing markers of common ancestry amongst taxa. Nevertheless, chromosomal rearrangements have been relatively under-utilised as evolutionary markers within phylogenetic investigations (Dobigny *et al*. 2004a). This is in part due to ambiguities in the identification of regions of chromosomal homology between taxa.

Chromosomal banding techniques, including G- and C-banding, allow for: a) the identification and characterisation of chromosomes, b) the detection of regions of chromosome homology, and c) the recognition of chromosomal rearrangements by means of banding polymorphisms. Comparative banding studies have revealed that karyotypic evolution within bats is dominated by Robertsonian (Rb) rearrangements (centric fusions or fissions), with inversions, reciprocal translocations, centromere shifts and heterochromatic additions (i.e. non-Rb translocations) reportedly being less common (Bickham 1979; Baker & Bickham 1980).

Traditional banding studies have limited use when comparing the chromosomes of taxa from divergent lineages or with radically reorganised genomes. Rearrangements that do not result in obvious differences in chromosomal banding patterns cannot be easily detected. Molecular cytogenetic techniques, such as Zoo-FISH (zoo-fluorescence *in situ* hybridisation) or cross-species chromosome painting using chromosome specific painting probes, however, allows for the resolution of homologous chromosomes / chromosomal arms and syntenies (i.e. conserved units of gene loci) between even distantly related taxa (Wienberg & Stanyon 1997; Ferguson-Smith & Trifinov 2007). Syntenic associations can represent chromosomal synapomorphies that allow for an independent determination of phylogenetic relationships amongst the taxa under study (Dobigny et al. 2004a). Shared syntenic associations are reportedly more useful in inferring evolutionary relationships than those rearrangements that result in disruptions of ancestral synteny (Robinson *et al*. 2008), as chromosomal breakpoints may not be identical across all taxa resulting in different combinations of two or more homologous elements (e.g. Volleth 2013).

To date approximately 50 bat species belonging to ten families have been studied using cross-species chromosome painting with chromosomal probes mostly derived from *Homo sapiens* (HSA), *Myotis myotis* (MMY), *Aselliscus stoliczkanus* (AST) or other bat probes (Volleth *et al*. 1999, 2002, 2013; Pieczarka *et al*. 2005; Ao *et al*. 2006, 2007; Eick *et al*. 2007; Mao *et al*. 2007, 2008, 2010; Richards *et al*. 2010; Kulemzina *et al*. 2011; Sotero-Caio *et al*. 2011; Table 1). Only three studies have incorporating species from Africa and the Malagasy subregions (Volleth & Heller 2007; Volleth *et al*. 2002; Eick *et al*. 2007; Table 1). Hence, our understanding of chromosomal evolution among Afrotropical bat lineages remains depauperate.

Table 1. Chiropteran species that have been studied using cross species chromosome painting techniques. The list is non-exhaustive as it does not include those species investigated in this study.

Family	Species	Region	Probe	Study
Pteropodidae	*Cynopterus sphinx*	Indomalayan	MMY	Ao *et al*. 2007
	Eonycteris spelaea	Indomalayan	HSA	Volleth *et al*. 2002
	Rousettus leschenaulti	Indomalayan	AST	Mao *et al*. 2007
Hipposideridae	*Aselliscus stoliczkanus*	Indomalayan	HSA, MMY	Mao *et al*.2007 ; Ao *et al*.2007
	Hipposideros armiger	Indomalayan	AST	Mao *et al*. 2010
	Hipposideros larvatus	Indomalayan	HSA, AST	Volleth *et al*. 2002 ; Mao *et al*. 2007
	Hipposideros pomona	Indomalayan	AST	Mao *et al*. 2010
	Hipposideros pratti	Indomalayan	AST	Mao *et al*. 2010
Rhinolophidae	*Rhinolophus affinis*	Indomalayan	AST	Mao *et al*. 2007
	Rhinolophus ferrumequinum	Indomalayan	AST	Mao *et al*. 2007
	Rhinolophus pearsoni	Indomalayan	AST	Mao *et al*. 2007
	Rhinolophus hipposideros	Palaearctic	MMY	Volleth *et al*. 2013
	Rhinolophus pusillus	Indomalayan	AST	Mao *et al*. 2007
	Rhinolophus rex	Indomalayan	AST	Mao *et al*. 2007
	Rhinolophus sinicus	Indomalayan	AST, MMY	Mao *et al*. 2007 ; Ao *et al*. 2007
	Rhinolophus meheleyi	Palaearctic	HSA, MMY	Volleth *et al*. 2002 ; Ao *et al*. 2007
Megadermatidae	*Megaderma spasma*	Indomalayan	HSA	Mao *et al*. 2008
Emballonuridae	*Taphozous melanopogon*	Indomalayan	HSA	Mao *et al*. 2008
Phyllostomidae	*Carollia brevicauda*	Neotropical	PHA	Pieczarka *et al*. 2005
	Phyllostomus hastatus	Neotropical	CBR	Pieczarka *et al*. 2005
	Desmodus rotundus	Neotropical	CBR, PHA	Sotero-Caio *et al*. 2011
	Diaemus youngi	Neotropical	CBR, PHA	Sotero-Caio *et al*. 2011
	Diphylla eucaudatus	Neotropical	CBR, PHA	Sotero-Caio *et al*. 2011
	Glossophaga soricina	Neotropical	HSA	Volleth *et al*.1999
Molossidae	*Tadarida teniotis*	Palaearctic	MMY	Mao *et al*. 2008
	Mops mops	Indomalayan	HSA	Volleth *et al*. 2002
	Mormopterus jugularis	Afrotropical	HSA	Volleth *et al*. 2002
	Mormopterus planiceps	Australasian	HSA	Volleth *et al*. 2002
Miniopteridae	*Miniopterus fuliginosus*	Indomalayan	MMY	Ao *et al*. 2006
Vespertilionidae	*Eptesicus bottae*	Palaearctic	HSA	Volleth *et al*. 2001
	Glauconycteris beatrix	Afrotropical	MMY	Volleth & Heller 2007
	Hesperoptenus blanfordi	Palaearctic	HSA	Volleth *et al*. 2001
	Hypsugo pulveratus	Indomalayan	MMY	Ao *et al*. 2006
	Murina hilgendorfi	Palaearctic	AST	Kulemzina *et al*. 2011
	Myotis altarium	Indomalayan	MMY, AST	Ao *et al*. 2006, Mao *et al*. 2007
	Myotis dasycneme	Palaearctic	HSA, AST	Volleth *et al*. 2002, Kulemzina *et al*. 2011
	Myotis myotis	Palaearctic	HSA	Volleth *et al*. 2002
	Nyctalus velutinus	Indomalayan	MMY	Ao *et al*. 2006
	Pipistrellus pygmaeus	Palaearctic	HSA	Volleth *et al*. 2002
	Plecotus auritus	Palaearctic	AST	Kulemzina *et al*. 2011
	Scotophilus dinganii	Afrotropical	MMY	Eick *et al*. 2007
	Scotophilus mhlanganii	Afrotropical	MMY	Eick *et al*. 2007
	Tylonycteris robustula	Indomalayan	MMY	Ao *et al*. 2006
	Tylonycteris sp.	Indomalayan	MMY	Ao *et al*. 2006
	Vespertilio murinus	Palaearctic	AST	Kulemzina *et al*. 2011

AST – *Aselliscus stoliczkanus*; CBR – *Carollia brevicauda*; PHA - *Phyllostomus hastatus*; HSA – *Homo sapiens*; MMY – *Myotis myotis*.

Painting studies have demonstrated that chiropteran chromosomal evolution is mostly characterised by conservation of whole syntenic blocks; typically whole chromosomes or chromosomal arms (karyotypic orthoselection), with a few exceptions (see review of Volleth & Eick 2012; Volleth 2013). FISH analyses also revealed that prevailing Rb rearrangements tend to produce identical arm combinations in both closely and distantly-related taxa (Mao *et al*. 2007, 2008). Identical chromosomal fusion products in distantly-related taxa may be a result of convergence (homoplasy) or, as in the case of intrafamilial karyotype evolution, it may indicate the retention of a chromosomal polymorphism through multiple speciation events (hemiplasy *sensu* Avise & Robinson 2008; Robinson *et al*. 2008). In some studies with wide-spread homoplasies and/or limited taxon sampling, chromosomal syntenies are mapped *a posteriori* onto an existing phylogeny (e.g. Mao *et al*. 2007, 2008; Richards *et al*. 2010; Sotero-Caio *et al*. 2011), and/or the cytogenetic signatures are interpreted within an evolutionary framework (e.g. Volleth *et al*. 2002; Ao *et al*. 2007). These approaches have been able to deduce chromosomal characters, including Rb products, which may be phylogenetically important.

Based on reciprocal painting studies using human and bat (*M. myotis*) probes, it was established that 25 chromosomal syntenic blocks or 'evolutionary conserved units' (ECUs following Volleth *et al*. 2002), have been retained during chiropteran chromosomal evolution (Table 2). These ECUs are reported in reference to HSA homology and have been found to sometimes vary in chromosome morphology amongst taxa (Volleth *et al*. 2002; Volleth *et al*. 2011). Variations in the structural appearance of the ECUs (e.g. centromere shifts, inversions, fissions and fusions), can represent valuable characters for phylogenetic inference. Eight syntenic associations of the presumed eutherian ancestral karyotype have been found in Chiroptera (see review of Volleth & Eick 2012; Table 1). A further seven HSA syntenies represent unique cytogenetic signatures for bats (Volleth *et al*. 2002; Table 1). Increase in available chromosome banding and painting data for Chiroptera will aid in the estimation of evolutionary rearrangements within different lineages and will provide a better understanding of the utility of these cytogenetic techniques in the reconstruction of the ancestral chiropteran chromosomal complement.

Table 2. Twenty-five evolutionary conserved units (ECUs: Volleth *et al*. 2002) that have characterised chiropteran chromosomal evolution. Chromosomal homologies between human and bat (*Myotis myotis*), as revealed by bi-directional painting are reported (Volleth *et al*. 2011). HSA syntenic associations are as reported in Ruiz-Herrera *et al*. (2012). Underlined syntenies are representative of the syntenic segmental associations for the postulated mammalian ancestor (Robinson & Ruiz-Herrera 2008; Ruiz-Herrera *et al*. 2012). [a] = bat-specific segment combinations.

Evolutionary conserved unit (ECU)	HSA homology	MMY homology
1a-6b[a]	1pter-p22, 6pter-p22	3
1b	1p13-q23, 1q23-q25, 1q32	22
1c	1q31, 1q41-qter	25
2a	2q14-qter	11
2b	2pter-q13	15
3a-21	3q12-q21, 3q23-q26, 3q27-qter, 21q	4
3b	3pter-p26, 3p25-p21, 3p21-p13, 3q22-q23	19
4a-10b[a]	4pter-p13, 4p12-q21, 4q22-q24, 4q25-q26, 10p	2
4b-8c-19b[a]	4q27-q31, 8p21, 8p23, 19p	7i
5a-7b-16b[a]	5pter-q23, 16p, 7q11.2, 7q21.3-q22	8
5b	4q32, 5q31-qter	7ii
6a	6p21-qter, 4q32	9
7a	7p21-q11.21, 7q11.23-q21.3, 7q22.1-qter	12
8a	8q	20
9	9pter-qter	14
10a	10q	18
11a	11pter-cen, 11q12-11q23	13
11b-22b-12b[a]	11q23-qter, 12q23.3-qter, 22q11.2-q12.3	23
12a-22aq	12pter-q23.3, 22q12.3-qter	6
13-8b-4c[a]	13q, 8p22, 8p21-p11, 4q33-qter	5
14a-15a-14b-15b	14q11-q32, 15q11-q24	1
15c	14q32.2-qter,15q25-qter	24
16a-19a	16q, 19q	16/17
17	17	21
18-20[a]	18pter-qter, 20pter-qter	10

The geometric morphometric revolution

Morphometrics, derived from the Greek words ‘morphē’ (shape) and ‘metron’ (measurement) is a sub-field of biometry. It refers to the class of methods providing quantitative description, comparison, analysis, and interpretation of biologically relevant size and/or shape variation patterns between biological forms (Rohlf 1990). Traditional morphometrics involves the application of univariate and multivariate statistical analyses to sets of linear (size) measurements of various specimen characters (Adams *et al*. 2004). These characters usually correspond to the distances between two identifiable points or landmarks on the surface of a particular object, such as specimen crania. Such variables rarely include information on both the size and shape of the organisms under study, except in those cases where measurements such as angles are included (Marcus & Corti 1996). As the geometrical relationships amongst the distance measurements are not accounted for, analyses of traditional morphometric distance data may have limited discriminating power.

Geometric morphometrics has revolutionised the field of morphometrics by providing a robust method for analysing relationships amongst taxa at various taxonomic levels, as it incorporates both size and shape components of morphological diversity. Developed in the late 1980's, geometric morphometrics utilises landmark coordinates, taken from digitised specimen images recorded in two or three dimensions (Adams *et al*. 2004). Differences in the landmark configurations of individual specimens and the consensus configuration (i.e. the averaged landmark configuration for all specimens examined), are representative of shape and size variation that may be visualised using thin plate splines (TPS). Thin plate splines are representations of the relative displacements of landmarks of a specimen allowing for a visual amplification of shape changes otherwise indistinguishable and difficult to describe using traditional morphometric data. They are powerful guides to the biological and functional interpretation of evolutionary diversification (Bookstein 1996) and may assist in the identification of novel morphological traits that can corroborate controversial phylogenies. Hence, geometric morphometric data can play an integral role in evolutionary biology and in the discovery of unique morphological characters and characters states.

The cranium has commonly been used as a source of morphometric data. It represents a complex and integrated structure composed of three evolutionary significant and partially independent units: the basicranium (cranial vault), neurocranium (braincase, auditory bullae and frontal, parietal and occipital regions), and orofacial module (orbital, nasal, oral and masticatory regions) (Hallgrimson *et al*. 2007a,b). These anatomical regions vary in terms of ontogeny, function, and phenotypic expression and are governed by various neutral and adaptive forces (Caumul & Polly 2005; Hallgrimssom *et al*. 2007a; Cardini & Elton 2008). Hence, the cranium can be a rich source of phylogenetic informative characters. Recent studies have demonstrated that the cranium, in particular the neurocranial unit, carries phylogenetic signal (e.g. Caumul & Polly 2005; Cardini & Elton 2008; Klingenberg & Gidaszewski 2010). This is most clearly observed between recently diverged taxa with sequence divergence of mtDNA between 5 and 10% (Polly 2003; Caumul & Polly 2005). In this context, recent investigations have found significant relationships between morphological divergences amongst taxa as determined from cranial geometric morphometric data and phylogenetic and/or phylogeographic patterns (e.g. Sztencel-Jabłonka e*t al*. 2009; Evin *et al*. 2008, 2011; Velazco *et al*. 2010; Taylor *et al*. 2012). Reconstructed morphometric patterns have proven to be useful correlates of molecular derived phylogenies.

RATIONALE AND SCOPE OF STUDY

The work described in this thesis represents a series of systematic studies of Afrotropical Chiroptera that were aimed at elucidating some evolutionary relationships at higher and lower taxonomic levels described below. The different studies focused specifically on taxa from the African subregion and/or Madagascar, for which there is a general paucity of comprehensive and/or resolved phylogenies. I employed an integrative approach based on the principles of cumulative evidence, and used cross species chromosome painting and geometric morphometric techniques to: a) evaluate different evolutionary hypotheses based on sequence data, and b) to explore cytogenetic and morphometric character evolution amongst the various taxa under study. As certain data sets are only informative at limited hierarchical levels (Wetterer *et al*. 2000), I used molecular cytogenetic approaches to address taxonomic incongruities at the familial, sub-familial and generic levels, whilst geometric morphometric data were used in investigations directed at the species level. Molecular cytogenetics and geometric morphometric approaches were chosen for this study as they have been relatively under-utilised in evolutionary studies of bats from Africa and Madagascar.

TAXONOMIC ISSUES ADDRESSED IN THIS STUDY

Family level ambiguities within Vespertilioniformes (Chapter two)

Uncertainty and controversy surrounds the phylogenetic positioning of the enigmatic and endemic Malagasy Myzopodidae within the Chiroptera. Depending on the data set and analytical methods employed, topologies retrieved from different studies are for the most part, in conflict or incapable of fully resolving the phylogenetic affinities of this enigmatic family. Molecular studies that have used a concatenation of three mitochondrial (12S rRNA, $tRNA^{Val}$, 16S rRNA; Van Den Bussche & Hoofer 2001) and/or two nuclear (RAG2 and dentin matrix protein 1, Hoofer *et al*. 2003; Van Den Bussche *et al*. 2003) genes were congruent in placing the Myzopodidae as the most basal Vespertilioniformes taxon and sister to Emballonuridae. Van Den Bussche *et al*. (2003) using RAG2 sequence data alone retrieved an alternate topology that positioned *Myzopoda* within the Emballonuridae. Further molecular analyses using a larger nuclear data set (PRCK1, SPTBN, STAT5A, THY) placed *Myzopoda* within the Vespertilionoidea, albeit with low support (Eick *et al*. 2005). More recently analyses of 17 introns from nuclear genes provided strongly supported evidence for the positioning of *Myzopoda* as the most basal within the largely Neotropical superfamily Noctilionoidea (Teeling *et al*. 2005; Miller-Butterworth *et al*. 2007). Based on these latter phylogenetic studies, the closest sister family to Myzopodidae was the New Zealand Mystacinidae. Until molecular consensus is reached regarding the true evolutionary affinities of Myzopodidae, topologies derived from

alternative data sets need to be explored in order to provide perspective regarding the true biogeographical origins of this family.

Genus level incongruities within Pteropodidae and Hipposideridae (Chapter three)

The Pteropodidae is the most diverse group of bats within the Pteropodiformes suborder, comprising 46 genera and 186 species (Simmons 2005). There are approximately 42 Afrotropical species, with 35 African species and a further seven species distributed throughout Malagasy subregion (ACR 2012). Andersen (1912), using morphological characters, formerly established the evolutionary framework of pteropodid classification: Macroglossinae (nectar- and pollen-feeders); Harpyionycterinae (containing the single genus *Harpyionycteris*); Pteropodinae (containing the remaining genera). The great morphological diversity within this group has made further traditional classifications based on cranial and anatomical characters, particularly difficult.

Bergmans (1997) refined pteropodid classification by incorporating findings from molecular studies using DNA-hybridisation and mtDNA sequence data, leading to the recognition of six subfamilies and nine tribes including the Afrotropical endemic Epomophorinae. In recent years several studies have attempted to resolve inconsistencies in the classification of pteropodids using molecular-based phylogenies. Studies based on mtDNA (Juste *et al*. 1999; Álvarez *et al*.1999) and concatenated nuclear and mtDNA data sets (Giannini & Simmons 2005; Almeida *et al*. 2011) are congruent in recognising a derived and monophyletic clade composed primarily of African genera within the Epomophorinae. This clade includes *Rousettus* and *Eonycteris*, (both genera formerly classified to Rousettinae *sensu* Bergmans 1997), to the exclusion of the African genus *Eidolon*. Inconsistencies regarding the phylogenetic affinities of the Cynopterinae (including the genus *Cynopterus*) and Pteropodinae (including the genus *Pteropus*) have hampered the interpretation of the evolutionary diversification and morphological adaptations within the family.

Based on mtDNA data alone, the Cynopterinae and Pteropodinae subfamilies have been regarded as sister clades that occupy a basal position relative to the Epomophorinae (Álvarez *et al*. 1999; Juste *et al*. 1999). Such associations are only weakly supported as mtDNA is, in some cases, unable to resolve deep lineage relationships (Galtier *et al*. 2009). Concatenated data sets of nuclear and mtDNA, using an increased taxonomic representation of fruit bat genera, have provided better-supported phylogenies (e.g. Almeida *et al*. 2011). It was shown that Cynopterinae is the most basal tribe and the Pteropodinae is the successive sister group to *Eidolon* (Almeida *et al*. 2011). In general, the rapid diversification of the group has resulted in some unresolved or weakly supported nodes of molecular phylogenies that make it difficult to determine basal relationships amongst pteropodid genera (Giannini & Simmons, 2003, 2005; Almeida *et al*., 2011).

Evolutionary associations amongst the nine genera and 81 species within the family Hipposideridae are another source of contention amongst Pteropodiformes taxa. In general, inter-generic relationships remain debatable as morphological and molecular based phylogenies are either poorly-resolved or are incongruent in assigning basal placement (Bogdanowicz & Owen 1998; Jones *et al*. 2002; Wang *et al*. 2003; Benda & Vallo 2009). The phylogenetic placement of the genera *Aselliscus* and *Hipposideros* have attracted the most attention in recent literature. Cladistic analyses of morphological data placed *Aselliscus* at the root of the hipposiderid tree (Hand & Kirsch 1998, 2003), which contradicts certain molecular DNA studies that revealed *Aselliscus* as nested within *Hipposideros* (Wang *et al*. 2003), or occupying the terminal branches of the hipposiderid tree (Li *et al*. 2007). The most recent and inclusive molecular investigation of hipposiderid genera, showed *Hipposideros* as the most basal lineage in clade containing the genera *Asellia*, *Coelops* and *Aselliscus* (Benda & Vallo 2009).

Evolutionary relationships amongst the most speciose hipposiderid genus, *Hipposideros* remain unresolved. Basal relationships amongst the numerous taxa remain in question as there is no comprehensive phylogeny for *Hipposideros* worldwide (Murray *et al*. 2012). The taxonomy of the more than 16 species described from the Afrotropics is unclear as many of the African forms have not yet been surveyed using molecular techniques (Vallo *et al*. 2008). Furthermore, recent genetic studies of certain taxa have begun to reveal high levels and cryptic diversity and paraphyly within several currently recognised *Hipposideros* spp. (Vallo *et al*. 2008, 2011a; Monadjem *et al*. 2013). Phylogenetic studies of limited taxa have suggested that the large Afrotropical endemics, that includes taxa formerly assigned to the *H. commersoni* group (*H. commersoni*, *H. gigas*, *H. thomensis*, *H. vittatus*), may occupy the basal positions within the genus tree and should therefore bear more primitive evolutionary traits (Eick *et al*. 2005; Vallo *et al*. 2008; Monadjem *et al*. 2013). This molecular hypothesis is yet to be tested using alternative data sets.

Species level incongruities within the genus *Otomops* (Chapter four)

Of the 17 genera within the family Molossidae, seven genera comprising 44 species are found within the Afrotropical region (Simmons 2005; ACR 2012). Whilst recent studies have provided some clarity regarding the diversity, biogeography and intra-generic relationships (see Ratrimomanarivo *et al*. 2007, 2008, 2009; Taylor *et al*. 2009), very little is understood of the intergeneric affiliations. Two recent studies have provided the first molecular insights into the biogeographical and temporal origins of the family (Lamb *et al*. 2011; Ammerman *et al*. 2012). Using mtDNA and nuclear sequence data from six genera and 17 species, Lamb *et al*. (2011) raised important questions regarding the taxonomic validity of certain generic and specific arrangements as inferred from previous traditional morphological classifications (Freeman 1981; Legendre 1984). For example RAG2 sequence data (Lamb *et al*. 2011) and mtDNA data (Lamb

et al. 2008) have revealed three distinct and reciprocally-monophyletic lineages of Afrotropical *Otomops*: a lineage from north-east Africa and Arabia, constituting an undescribed taxon; a clade from sub-Saharan Africa (excluding north-east Africa), referable to *O*. *martiensseni*; and a taxon from Madagascar referable to *O*. *madagascariensis*. This is in conflict with traditional classifications based upon morphological data that either recognise both a single polytypic Afro-Arabian species (*O*. *martiensseni*) and separate Malagasy species (*O*. *madagascariensis*) (Simmons 2005), or three distinct taxa, namely, *O. martiensseni* from east Africa, *O*. *icarus* from southern and central Africa, and *O*. *madagascariensis* from Madagascar (Peterson *et al*. 1995). The discordance between the morphological and genetic delineation of Afrotropical *Otomops* and the possibility of a new species from the African subregion warrant a detailed assessment of the morphological diversity of *Otomops*. Further studies with increased sample sizes and using alternative methods are required to resolve the current taxonomic uncertainty regarding Afrotropical *Otomops*.

GENERAL AIMS AND OBJECTIVES

This study used two approaches to investigate taxonomic uncertainties amongst Afrotropical Chiroptera. The aims of the molecular cytogenetic component of the study were focussed on the analysis of karyotypic diversity and evolution amongst Malagasy bats belonging to both the Vespertilioniformes and Pteropodiformes suborders. Novel G- and C-band data for several species were presented and used in combination with unidirectional cross species chromosome painting utilising *Myotis myotis* (MMY) painting probes, to generate genome-wide comparative maps of the taxa under study. Identified chromosomal rearrangements and syntenies were used to:

1. Define the mode (i.e. type of chromosomal rearrangements) and possible role of chromosomal evolution in the evolutionary history of Afrotropical Chiroptera.
2. Test recent molecular-based hypotheses regarding the evolutionary placement of the family Myzopodidae by mapping chromosomal rearrangements onto a molecular based phylogeny (Teeling *et al*. 2005; Miller-Butterworth *et al*. 2007) (Chapter Two).
3. Evaluate the phylogenomic relationships between the Pteropodinae and Epomophorinae subfamilies (Chapter Three)
4. Assess the validity of recently-described chromosomal synapomorphies for the families Pteropodidae and Hipposideridae (Chapter Three).
5. Reassess the phylogenomic positioning of the genus *Hipposideros* within the family tree using chromosome painting data for Malagasy *H*. *commersoni* and compare these relationships to existing systematic classification for hipposiderids (Chapter Three).

The second focus of the study was directed at evaluating and characterising morphological divergence between geographical populations of Afrotropical *Otomops* and to test the taxonomic validity of recently described genetic lineages (Lamb *et al*. 2008). Geometric morphometric data (landmarks) described from the crania of *Otomops* were used to:

1. Assess the congruence between morphological and genetic patterns of diversity. (Chapter Four)
2. Establish the species limits of *Otomops* taxa from sub-Saharan Africa, the Arabian Peninsula, and Madagascar (Chapter Four)
3. Evaluate the adaptive significance of morphological evolution amongst *Otomops* from Africa and the Arabian Peninsula (Chapter Four).

ARRANGEMENT AND STYLE OF THESIS

Most of the work presented in this thesis has been published. This thesis has been prepared according to the format of manuscripts for publication in peer-reviewed journals. This has resulted in variation in the format of the three research chapters and some repetitive text particularly in the case of the research method sections. Included in each chapter is an introduction to the content under discussion, a brief description of the methods and analytical techniques utilised, results and discussion sections, and appendices. Figures and Tables are labelled according to the relevant chapters in which they appear and not for the complete thesis. Pages are numbered sequentially and a comprehensive list of references is provided at the end of the dissertation.

Chapter 1 – General introduction

Chapter 2 – Superfamily and family level investigations

Richards LR, Rambau RV, Lamb JM, Taylor PJ, Yang F, Schoeman MC, Goodman SM (2010) Cross-species chromosome painting in bats from Madagascar: the contribution of Myzopodidae to revealing ancestral syntenies in Chiroptera. *Chromosome Research* **18:** 635–653.

Chapter 2 – Family and genus level investigations

Richards LR, Rambau RV, Goodman SM, Taylor PJ, Schoeman MC, Lamb JM (manuscript) Karyotypic evolution in Malagasy flying foxes (Pteropodidae, Chiroptera) and their rhinolophoid relatives as determined by comparative chromosome painting.

Chapter 4 – Species level investigations

Richards LR, Taylor PJ, Schoeman MC, Goodman SM, Van Daele PAAG, Lamb JM (2012) Cranial size and shape variation in Afrotropical *Otomops* (Mammalia: Chiroptera: Molossidae): testing species limits using a morphometric approach. *Biological Journal of the Linnean Society* **106:** 910–925.

Chapter 5 – Summary and concluding comments

CHAPTER TWO

SUPER-FAMILY AND FAMILY LEVEL INVESTIGATIONS [1]

[1] For consistency throughout the thesis, information in the following text has been updated since the 2010 publication.

CROSS-SPECIES CHROMOSOME PAINTING IN BATS FROM MADAGASCAR: THE CONTRIBUTION OF MYZOPODIDAE TO REVEALING ANCESTRAL SYNTENIES IN CHIROPTERA

Leigh R. Richards[1], Ramugonelo V. Rambau[2*], Jennifer M. Lamb[1], Peter J.Taylor[4,5], F. Yang[3], M. Corrie Schoeman[1], Steven M. Goodman[6,7]

[1] School of Biological and Conservation Sciences, University of KwaZulu-Natal, Westville Campus, Durban, 4001, South Africa

[2] DST-NRF Center of Excellence for Invasion Biology and Evolutionary Genomics Group, Department of Botany and Zoology, University of Stellenbosch, Stellenbosch, South Africa, Private Bag X1, Matieland, 7602, South Africa*

[3] The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SA United Kingdom

[4] Durban Natural Science Museum, P O Box 4085, Durban, 4000, South Africa

[5]School of Environmental Sciences, University of Venda, Private Bag X5050, Thohoyandou, 0950

[6] Field Museum of Natural History, Department of Zoology, 1400 S Lake Shore Drive, Chicago, IL 60605 USA;

[7] Vahatra, BP 3972, Antananarivo 101, Madagascar

KEYWORDS Chiroptera, Madagascar, *Myzopoda*, chromosome painting, karyotypic evolution

ABBREVIATIONS

CBG-banding	C-banding by treatment with barium hydroxide
GTG-banding	G-banding by trypsin digestion
IHB	Intercalary heterochromatic block
MAU	*Myzopoda aurita*
MGO	*Myotis goudoti*
MGR	*Miniopterus griveaudi*
MJU	*Mormopterus jugularis*
MMY	*Myotis myotis*
Rb	Robertsonian
X-A	X-autosome translocation
Zoo-FISH	Zoo-fluorescence *in situ* hybridization

ABSTRACT

The chiropteran fauna of Madagascar comprises eight of the 20 recognized families of bats, including the endemic Myzopodidae. While recent systematic studies of Malagasy bats have contributed to our understanding of the morphological and genetic diversity of the island's fauna, little is known about their cytosystematics. Here we investigate karyotypic relationships among four species, representing four families of Chiroptera endemic to the Malagasy region using cross-species chromosome painting with painting probes of *Myotis myotis*: Myzopodidae (*Myzopoda aurita*, 2n=26), Molossidae (*Mormopterus jugularis*, 2n=48), Miniopteridae (*Miniopterus griveaudi*, 2n=46), and Vespertilionidae (*Myotis goudoti*, 2n=44). This study represents the first time a member of the family Myzopodidae has been investigated using chromosome banding and chromosome painting techniques. Painting probes of *M. myotis* were used to delimit 29, 24, 23, and 22 homologous chromosomal segments in the genomes of *M. aurita*, *M. jugularis*, *M. griveaudi*, and *M. goudoti*, respectively. Comparison of GTG-banded homologous chromosomes/chromosomal segments among the four species revealed the genome of *M. aurita* has been structured through 14 fusions of chromosomes and/or chromosomal segments homologous to *M. myotis* chromosomes leading to a karyotype consisting solely of bi-armed chromosomes. In addition, chromosome painting revealed a novel X-autosome translocation in *M. aurita*. Comparison of our results with published chromosome maps provided further evidence for karyotypic conservatism within the genera *Mormopterus*, *Miniopterus*, and *Myotis*. Mapping of chromosomal rearrangements onto a molecular consensus phylogeny revealed chromosomal syntenies shared between *Myzopoda* and other bat species of the infraorders Pteropodiformes and Vespertilioniformes. Our study provides further evidence for the involvement of Robertsonian (Rb) translocations and fusions/fissions in chromosomal evolution within Chiroptera.

INTRODUCTION

Madagascar is home to eight of the 20 recognized chiropteran families, of which two belong to the suborder Pteropodiformes (Pteropodidae and Hipposideridae) and six to the suborder Vespertilioniformes (Emballonuridae, Miniopteridae, Molossidae, Myzopodidae, Nycteridae, and Vespertilionidae) (Goodman 2011; ACR 2012). Until a decade ago, the systematics and biogeographical affinities of the Malagasy bat fauna remained poorly known (Peterson et al. 1995; Eger and Mitchell 1996, 2003). Recent biological surveys and systematic studies utilizing morphometric and/or molecular sequencing techniques have refined our knowledge of the evolutionary relationships among bats; consequently the number of Malagasy Chiroptera has

increased from 28 species and 19 genera (Eger and Mitchell 2003) to over 44 species belonging to 23 genera, and a species-level endemism of approximately 70% (Goodman 2011).

Of these endemics, the family Myzopodidae (represented by *Myzopoda aurita* and *M. schliemanni*), is the most enigmatic with regard to its phylogenetic position, which has fluctuated among three currently recognized superfamilies: Vespertilionoidea (Koopman 1994b; Eick et al. 2005), Emballonuroidea (Van Den Bussche et al. 2003), and Noctilionoidea (Teeling et al. 2005; Miller-Butterworth et al. 2007). Cladistic analyses of morphological data place *Myzopoda* either basal to the Nataloidea (sensu Simmons 1998) and Vespertilionoidea (Smith 1976), within the Nataloidea (Simmons and Geisler 1998), or within the Vespertilionoidea (Koopman 1994b). In contrast, molecular studies using a concatenation of three mitochondrial (12S rRNA, tRNAval, 16S rRNA; Van Den Bussche and Hoofer 2001) and/or two nuclear genes (RAG2 and dentin matrix protein 1, Hoofer et al. 2003; Van Den Bussche et al. 2003) are congruent in placing the Myzopodidae as the most ancestral family within the Vespertilioniformes, sister to Emballonuridae.

Using only RAG2 sequence data, Van Den Bussche et al. (2003) retrieved an alternate topology positioning *Myzopoda* within the Emballonuridae. Further molecular analyses based on nuclear markers (PRCK1, SPTBN, STAT5A, THY), position *Myzopoda* within the Vespertilionoidea (Eick et al. 2005). More recently, analyses of 17 introns from nuclear genes placed *Myzopoda* as the most basal member of the largely Neotropical superfamily Noctilionoidea, with the closest sister family being the New Zealand Mystacinidae (Teeling et al. 2005, Miller-Butterworth et al. 2007). Following the phylogenetic hypothesis of Teeling et al. (2005) and Miller-Butterworth et al. (2007), *Myzopoda* originated from a Neotropical noctilionid ancestor that dispersed to Madagascar from South America during the early Eocene. This scenario is in stark contrast to recent phylogeographic studies on Malagasy bats which demonstrate colonization from Africa across the Mozambique Channel (Russell et al. 2008; Ratrimomanarivo et al. 2007, 2008) or from Asia (Lamb et al. 2008; O'Brien et al. 2009).

Chromosomal data have contributed significantly to our understanding of evolutionary relationships within and among chiropteran families. Comparative cytogenetic analyses have revealed that chromosome evolution in bats is largely conservative (Baker and Bickham 1980). For example, 65 of the 99 karyologically examined members of the Molossidae share a karyotype of 48 chromosomes (Sreepada et al. 2008). This karyotypic conservatism is also observed at the generic level, as in *Myotis* taxa where species typically exhibit diploid numbers of 2n=44 (Baker and Patton 1967; Bickham 1979a, b; Bickham et al. 1986). Intergeneric variation in diploid number of bats is usually mediated by Robertsonian (Rb) translocations and is characterized by centric fusions or fissions of whole chromosomal arms (Baker and Bickham 1980). While G-banding allows for easy identification of Rb rearrangements between species and/or genera of the same family, the use of Zoo-fluorescence *in situ* hybridization (Zoo-FISH) in combination with GTG-banding provides more detailed comparisons between taxa (Wienberg and Stanyon 1997; Ferguson-Smith and Trifonov 2007).

To date, approximately 50 species representing nine of the 20 global chiropteran families have been studied using cross-species chromosome painting (Volleth et al. 1999, 2001, 2002, 2013; Pieczarka et al. 2005; Ao et al. 2006, 2007; Eick *et al*. 2007; Volleth and Heller 2007; Mao et al. 2007, 2008, 2010; Kulemzina et al. 2011; Sotero-Caio et al. 2011, Volleth et al. 2013). With the exception of Volleth and Heller (2007) and Volleth et al. (2002), none of these studies have included representative species from Madagascar. Hence, the cytosystematics of Malagasy bats relative to those from other regions of the world is largely unknown. Herein we present genome-wide comparative chromosomal maps of four species of Malagasy bats generated using *Myotis myotis* flow-sorted chromosomes. These species represent four families: Myzopodidae (*Myzopoda aurita*), Molossidae (*Mormopterus jugularis*), Vespertilionidae (*Myotis goudoti*), and Miniopteridae (*Miniopterus griveaudi*); of these, the first three are endemic to Madagascar while the last occurs on Madagascar and the Comoros (Weyeneth et al. 2008; Goodman et al. 2009a). The karyotype of each species is presented here for the first time and compared with those of other species from the same families and/or superfamilies. We have now increased the taxon sampling in chromosome painting studies of the Chiroptera to 10 of the 20 recognized chiropteran families. Our aim was twofold. Firstly, we investigated karyotypic evolution among four Malagasy chiropteran families relative to other bat species using GTG-banding and chromosome painting based on *M. myotis* painting probes. Secondly, we test recent molecular-based hypotheses regarding the phylogenetic placement of *M. aurita* by mapping chromosomal rearrangements identified from published chromosomal maps of representatives of the superfamilies Vespertilionoidea (Volleth et al. 2002; Ao et al. 2006; Mao et al. 2008; this study), Emballonuroidea (Mao et al. 2008), and Noctilionoidea (Volleth et al. 1999) onto a molecular-based phylogeny (Teeling et al. 2005; Miller-Butterworth et al. 2007). This consensus phylogeny revealed wide-scale homoplasies between the Myzopodidae and several bat families. Our results provide further insights into the karyotypic evolution amongst Chiroptera and support previous studies suggesting the involvement of Rb fusions in genome restructuring of Chiroptera. Furthermore, we describe a novel X-autosome translocation identified in *M. aurita* using *M. myotis* whole chromosome probes.

MATERIALS AND METHODS

Specimens examined

The four species examined in this study were captured from natural habitats in eastern and western Madagascar using mist nets and harp traps (see Table 1). Specimens were identified using external morphological characters (e.g., tragus shape in the case of *Miniopterus*, Goodman et al. 2009a, b; Goodman 2011), and thereafter euthanized according to ethical guidelines of the American Society of Mammalogists (Sikes and Gannon 2011) and with the

approval of the Animal Ethics Committee of the University of KwaZulu-Natal, Westville Campus, South Africa. Voucher specimens, identified by SMG, were deposited in the Field Museum of Natural History, Chicago.

Table 1. Bat species investigated in this study.

Scientific name and abbreviation	Locality	GPS coordinates	Number and sex	2n	FNA	Accession number
Myzopoda aurita (MAU)	Station Forestiere d'Ivoloina, Province de Toamasina, Madagascar	18°05702 S, 49°35908 E	1 ♀	26	48	FMNH 194176
	Forêt de Sahafina, Province de Toamasina, Madagascar	18°81027 S, 49°98000 E	1♀	26	48	SMG 16636
Mormopterus jugularis (MJU)	Grotte d'Ambanilia, Province de Toliara, Madagascar	23°54000 S, 43°74611 E	1 ♂	48	54	FMNH 202492
Miniopterus griveaudi (MGR)	Grotte d' Anjohibe, Province de Mahajanga, Madagascar	15°32289 S, 46°53159 E	1 ♀	46	50	SMG 16249
Myotis goudoti (MGO)	Grotte d'Ambanilia, Province de Toliara, Madagascar	23°54000 S, 43°74611 E	1♂	44	50	FMNH 202490
	Forêt de Maromizaha, Region Alaotra Mangoro, Madagascar	18°98138 S, 48°46388 E	1♀	44	50	SMG 16191

FMNH Field Museum of Natural History, Chicago, *SMG* field collection number of Steven M. Goodman in cases when final catalog numbers in the FMNH have yet to be assigned.

Chromosome preparation and GTG- and CBG-banding

Metaphase spreads were harvested either from bone marrow preparations following Volleth et al. (2009), or from fibroblast cell cultures established from tail- and wing-membrane explants using standard cytogenetic protocols. GTG-banding was obtained following Seabright (1971) with slight modifications. Metaphase chromosome slides were digested in a 0.025% trypsin solution for 10–30 s, followed by two rinses in fetal calf serum buffer (500 µl FCS in 50 ml phosphate buffer), and then stained with a 10% Giemsa solution for 4–5 min. CBG-banding was carried out using the method of Sumner (1972), wherein slides were initially treated with a 0.2 M HCl solution for 3 min and then incubated in a 5% Ba(OH)2 solution at 55℃ for 1–2 min. Following this, slides were incubated for 30–45 min in a 2× SSC solution at 55℃ and stained for 5–8 min using a 10% Giemsa solution.

Zoo-FISH

In order to detect regions of homology among chromosomes of the four species analysed in this investigation, we used the complete suite of *M. myotis* whole chromosome painting probes (21 whole chromosome painting probes representing 21 *M. myotis* (MMY) autosomes and the X chromosome, Ao et al. 2006). Flow-sorted MMY probes have been successfully used in past chromosome painting studies of the families Molossidae, Vespertilionidae, and Miniopteridae (see Ao et al. 2006 and Mao et al. 2008). Chromosome-specific painting probes were produced

using degenerate oligonucleotide PCR (DOP-PCR, Telenius et al. 1992) of flow-sorted chromosomes of *M. myotis* as previously described (Ao et al. 2006). *Myotis* probes were labelled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche Molecular Chemicals) by a secondary DOP-PCR amplification. Probe DNA was precipitated overnight at –80°C in a mixture comprising 6–8 µl DOP-PCR product, 6 µl salmon sperm DNA, 6 µl mouse Cot 1 DNA (in the case of *M. aurita*), 4 µl Na Acetate (3 M), and 100% ethanol. The precipitated probe mixture was centrifuged for 30 min, washed with 70% ethanol (at 4°C), pelleted, and air dried. Probes were dissolved in 15 µl hybridization buffer (50% deionized formamide, 10% dextran sulfate, 0.5 M phosphate buffer pH7.3, 1× Denhardt's solution). Probes were denatured for 10 min at 72°C and pre-annealed by incubation for 25–30 min at 37°C. Metaphase spreads were denatured at 65–67°C in 70% formamide/2× SSC for 1 min, rinsed in ice-cold 70% ethanol to halt the denaturation process, and finally dehydrated in an ethanol series and air dried. Pre-annealed probes were applied onto slides and allowed to hybridize at 37°C for 72 h. Biotin-labelled *Myotis* probes were detected using Cy3-labeled streptavidin (1:500 dilution, Amersham) and Dig-labelled probes were detected with anti-dig FITC (1:500 dilution, Amersham). Post-hybridization washes of slides included two washes in 50% formamide/2× SSC, two rinses in 2× SSC, and a wash in 4× SSC/0.1% Tween 20 at 42.5°C. This was followed by three 5 min washes in 4× SSC/0.1% Tween 20 at 37°C, after which slides were counterstained with 4-,6-diamidino-2-phenylindole (DAPI) for 10 min and mounted with an antifade reagent (Vectashield, Vector Laboratories).

Image capture and data processing

FISH images were captured using the Genus System version 3.7 (Applied Imaging Corp., Newcastle, UK) with a CCD camera mounted on an Olympus BX 60 epifluorescence microscope (Fig. 1). Hybridization signals were assigned to specific chromosomes or chromosomal segments as identified using enhanced DAPI-banding patterns resembling GTG-banding patterns.

Chromosome nomenclature and terminology

The karyotype of *M. goudoti* was arranged following the numbering convention of Bickham (1979a), where the chromosomal arms rather than individual chromosomes were numbered. The GTG-banded karyotype of *M. jugularis* was arranged following the numbering scheme for *Mormopterus planiceps* by Volleth et al. (2002), where bi-armed chromosomes are numbered first. Chromosomes of *M. griveaudi* were arranged according to the chromosomal complement of *Miniopterus fuliginosus* published by Ao et al. (2006), while the *M. aurita* karyotype was arranged according to relative chromosome size, from largest to smallest.

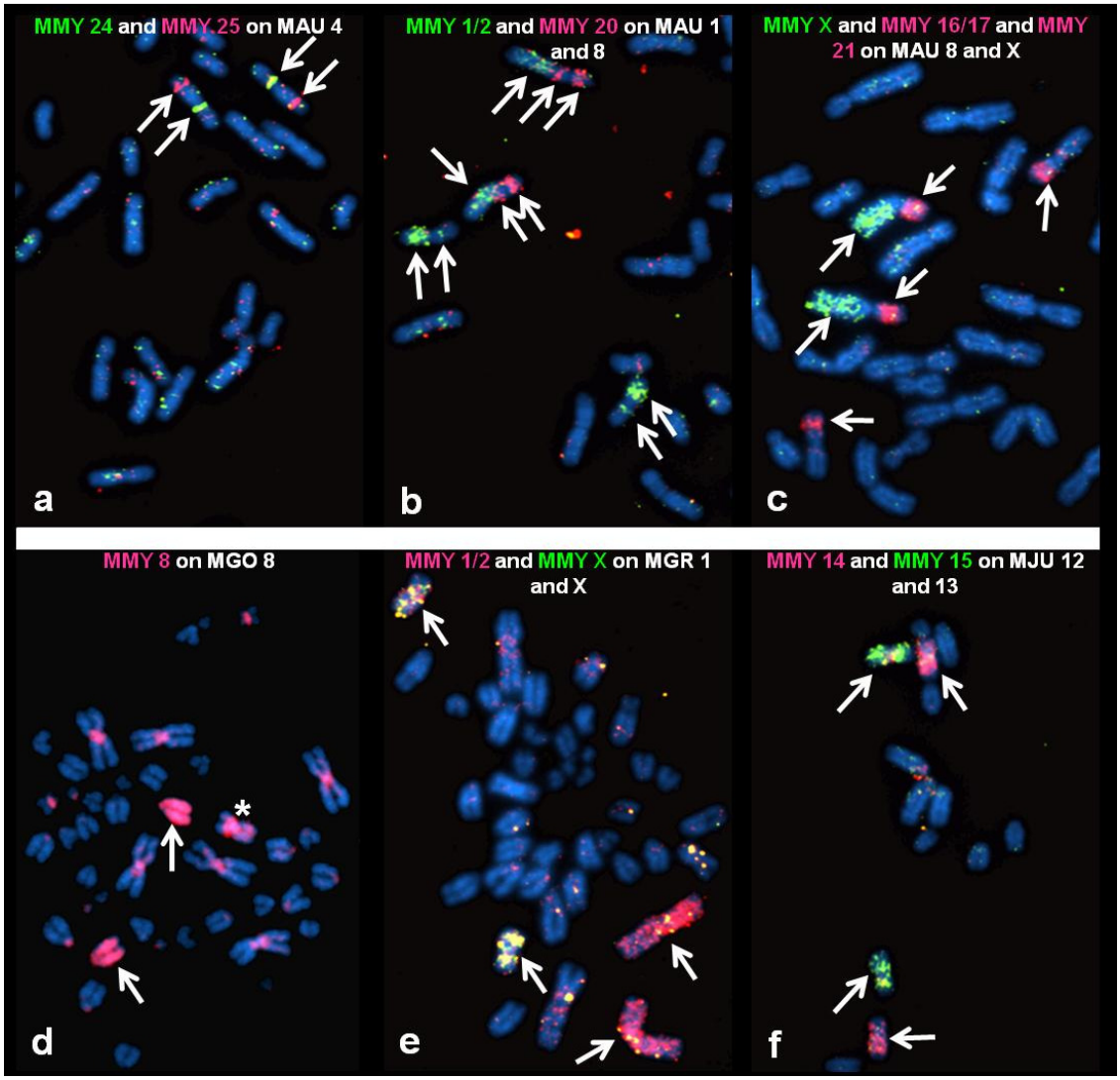

Figure 1. Examples of FISH results employing MMY probes indicated by cy3 (*red*) and FITC (*green*) signals on partial metaphase spreads of *Myzopoda aurita*, *Myotis goudoti*, *Miniopterus griveaudi*, and *Mormopterus jugularis*, which were counterstained using DAPI (*blue*). *White arrows* indicate hybridization signals on chromosomal regions/arms. **a** MMY24 and MMY25 hybridized to separate chromosomal arms of MAU4. **b** MMY1/2 hybridization to MAU1q and MAU8 (q arm and proximal portion of the p arm) indicating fission of MMY1/2 and hybridization of MMY20 to MAU1p and the proximal portion of MAU1q. Thus, MAU1 is a product of a fusion event between MMY20 and MMY1/2. **c** Hybridization of MMY21 and MMY X to the X chromosome of *M. aurita*. Therefore, the X chromosome of *M. aurita* is a composite chromosome formed as result of a sex-autosome translocation. **d** Chromosome painting of MMY8 on *M. goudoti* chromosome 8 representing the high degree of homology between *Myotis myotis* (2n=44) and *M. goudoti* (2n=44). The asterisk indicates background hybridization on the X chromosome of *M. goudoti*. **e** Conservation of MMY1/2 and MMY X on *M. griveaudi* chromosomes 1 and X, respectively. **f** Hybridization of MMY14 and 15 to *M. jugularis* chromosomes 12 and 13, respectively.

Table 2. Chiropteran species used in the mapping analysis. Species include seven representatives of the Pteropodiformes and 11 representatives of the Vespertilioniformes.

Family	Species and abbreviation	2n	Painting probe	Reference
Pteropodidae	*Eonycteris spelaea* (ESP)	36	HSA	Volleth *et al.* 2002
	Rousettus leschenaulti (RLE)	36	AST	Mao *et al.* 2007
Rhinolophidae	*Rhinolophus pearsoni pearsoni* (RPE)	44	AST	Mao *et al.* 2007
	Rhinolophus sinicus (RSI)	36	AST	Mao *et al.* 2007
Hipposideridae	*Aselliscus stoliczkanus* (AST)	30	HSA	Mao *et al.* 2007
	Hipposideros larvatus (HLA)	32	AST, HSA	Mao *et al.* 2007, Volleth *et al.* 2002
Megadermatidae	*Megaderma spasma* (MSP)	38	HSA	Mao *et al.* 2008
Emballonuridae	*Taphozous melanopogon* (TME)	42	HSA	Mao *et al.* 2008
Phyllostomidae	*Glossophaga soricina* (GSO)	32	HSA	Volleth *et al.* 1999
Myzopodidae	*Myzopoda aurita* (MAU)	26	MMY	This study
Molossidae	*Mormopterus jugularis* (MJU)	48	HSA, MMY	Volleth *et al.* 2002; This study
	Mormopterus planiceps (MPL)	48	HSA	Volleth *et al.* 2002
	Tadarida teniotis (TTE)	48	MMY	Mao *et al.* 2008
Miniopteridae	*Miniopterus fuliginosus* (MFE)	46	MMY	Ao *et al.* 2006
	Miniopterus griveaudi (MGR)	46	MMY	This study
Vespertilionidae	*Myotis altarium* (MAL)	44	MMY	Ao *et al.* 2006
	Myotis goudoti (MGO)	44	MMY	This study
	Myotis myotis (MMY)	44	HSA	Volleth *et al.* 2002

AST – *Aselliscus stoliczkanus*; HSA – *Homo sapiens*; MMY – *Myotis myotis*

Phylogenomic comparisons using chromosomal characters

In order to interpret our results in the context of other bat species, we compared our data with the published chromosome map data of an additional 14 species (Volleth et al. 1999, 2002; Ao et al. 2006, 2007; Mao et al. 2007, 2008; Table 2). We used the chromosome complement of *M. myotis* as a reference to delimit syntenic associations (Table 3), which were compiled using previously published chromosome painting data presented in Ao et al. (2007, Table 1) and Mao et al. (2007, Table 2; 2008, Table 2). Further, following the precedent in Ao et al. (2006) and Mao et al. (2007), we use *Myotis altarium* as a substitute for *M. myotis*, as the two species have similar karyotypes (Ao et al. 2006). Identified syntenic associations/disruptions were scored as present or absent in binary format. A total of 79 characters, including 73 fusion events and six MMY chromosome fissions were scored from 18 chiropteran taxa (Table 3). We mapped these characters onto relevant lineages of a DNA sequence-based phylogeny of higher level chiropteran systematics (Teeling et al. 2005; Miller-Butterworth et al. 2007). This allowed us to plot the polarity of karyotypic evolution among the various bat families unambiguously and to identify plesiomorphic and synapomorphic chromosomal rearrangements associated with the karyotypic evolution of *Myzopoda*.

Table 3. Data matrix of 79 chromosomal characters (syntenic associations of homologous chromosomes/chromosomal segments of *Myotis myotis*) mapped onto the consensus sequence-based tree (Teeling et al. 2005; Miller-Butterworth et al. 2007).

No.	Character	ESP	RLE	AST	HLA	RPE	RSI	MSP	TME	GSO	MAU	MAL	MGO	MMY	MFI	MGR	MJU	MPL	TTE
1	1/2	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1
2	1/3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
3	1/6	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
4	1/11	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
5	1/14	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	1/16/17	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
7	1/18	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	2/6	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
9	2/9	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	2/20	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
11	2/25	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	3/4	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0
13	3/7	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
14	3/9	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	3/11	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	3/13	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
17	3/15	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
18	3/18	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
19	4/5	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
20	4/6	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
21	4/8	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
22	4/14	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
23	4/18	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
24	4/19	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25	5/6	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0
26	5/8	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
27	5/10	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
28	5/14	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
29	5/23	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
30	6/10	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
31	6/11	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32	6/13	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
33	6/15	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
34	7/9	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
35	7/10	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
36	7/15	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
37	7/18	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
38	7/19	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
39	7/21	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
40	7/22	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
41	7/25	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
42	7/8/12/15	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
43	7/8/22/24/25	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
44	8/10	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
45	8/11	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
46	8/12	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
47	8/14	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
48	8/15/19	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
49	8/24	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	9/11	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0

Table 4. (continued)

No.	Character	ESP	RLE	AST	HLA	RPE	RSI	MSP	TME	GSO	MAU	MAL	MGO	MMY	MFI	MGR	MJU	MPL	TTE
51	9/15	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
52	9/19	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
53	10/12	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
54	10/18	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
55	10/24	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
56	11/16/17	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
57	11/20	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
58	12/14	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
59	12/16/17	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
60	12/25	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
61	13/14	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
62	13/15	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
63	13/23	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
64	15/21	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
65	16/17/24	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
66	18/21	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
67	18/23	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
68	19/20	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
69	20/22	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
70	20/25	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
71	21/22	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
72	21/25	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
73	21/X	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
74	Fi 7	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
75	Fi 8	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0
76	Fi 10	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0
77	Fi 12	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
78	Fi 20	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
79	Fi 22	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0

RESULTS

Karyotype analysis

(a) *M. aurita*: This is the first description of the karyotype of *M. aurita*, and has a diploid number of 2n=26 (NFa=48, Fig. 2a). All autosomes are bi-armed; these consist of one large submetacentric (pair 1), six metacentrics (pairs 2, 6, 7, 8, 9, and 11), and five submetacentric chromosomes (pairs 3, 4, 5, 10, and 12). The X chromosome is submetacentric. CBG-banding analysis revealed the presence of heterochromatin located within the centromeric and telomeric regions of all autosomes (Fig. 2a). Interstitial heterochromatin was detected in six autosomes (pairs 2–6 and 9) and on the proximal portion of the short arm of the X chromosome.

(b) *M. jugularis*: This species has a karyotype with a diploid number of 2n=48 (NFa=54). The chromosomal complement comprises a large metacentric (pair 1), three smaller metacentrics (pairs 2–4), 19 acrocentric autosomes (pairs 5–23), a submetacentric X, and a small

metacentric Y chromosome (Fig. 2b). Heterochromatin was detected in the terminal segments of the four metacentric chromosomes (data not shown) and interstitial regions in four acrocentric pairs (5, 6, 7, and 8).

(c) *M. griveaudi*: The karyotype of *M. griveaudi* has a diploid number of 2n=46 (NFa=50, Fig. 3a). It comprises two large metacentrics (pairs 1 and 2), one medium metacentric (pair 7), 19 acrocentric autosomes (pairs 3–6 and 8–22), and a submetacentric X. C-banding revealed the presence of heterochromatin localized within the centromeric regions of all chromosomes (data not shown).

(d) *M. goudoti*: The karyotypes of both individuals of this species have a diploid number of 2n=44 (NFa=50) and comprise three large metacentrics (pairs 1/2, 3/4, and 5/6), one small metacentric (pair 16/17), and 16 acrocentric autosomes (pairs 7–15 and 18–25; Fig. 3b). The X chromosome is a submetacentric, while the Y chromosome is a small acrocentric. Heterochromatin was present only in the centromeric regions of chromosomes, with the exception of chromosomes 7 and 8, which contained segments of interstitial heterochromatin below the centromere (C-banding data not shown).

Chromosome painting in four Malagasy bat species

Chromosome-specific MMY painting probes delimited 29 homologous chromosomal segments in the genome of *M. aurita* (Table 4 and Fig. 2a). Three MMY probes (MMY 3/4, 12, 13) were retained on single chromosomes corresponding to pairs MAU2, 11, and 12, respectively. Five chromosome pairs of *M. aurita* corresponded to two probes of *M. myotis*: MAU3 bore homology to MMY10 and 6; MAU6 corresponded to MMY9 and 11; MAU7 hybridized with MMY5 and 14; MAU9 corresponded to MMY18 and 23; and the X chromosome hybridized with MMY X and presumably MMY21 (as determined by differential DAPI-banding patterns used to discriminate between MMY16/17 and MMY21, which flow-sort together). Four MMY probes each hybridized to two separate *Myzopoda* autosomes: MAU8 and 1q hybridized with MMY1/2; MAU4p (dist) and 10q corresponded to MMY7; MAU4q (prox) and 5q (prox) were painted with MMY8; and MAU4p and 10q hybridized with MMY22. Furthermore, autosome 4 hybridized with three additional MMY probes (22, 24, and 25; Table 4 and Fig. 2a), whereas MAU5 hybridized with a further two whole chromosome probes (MMY15 and 19; Table 4, Fig. 2a). A total of 14 fusion events were detected in the karyotype of *M. aurita*.

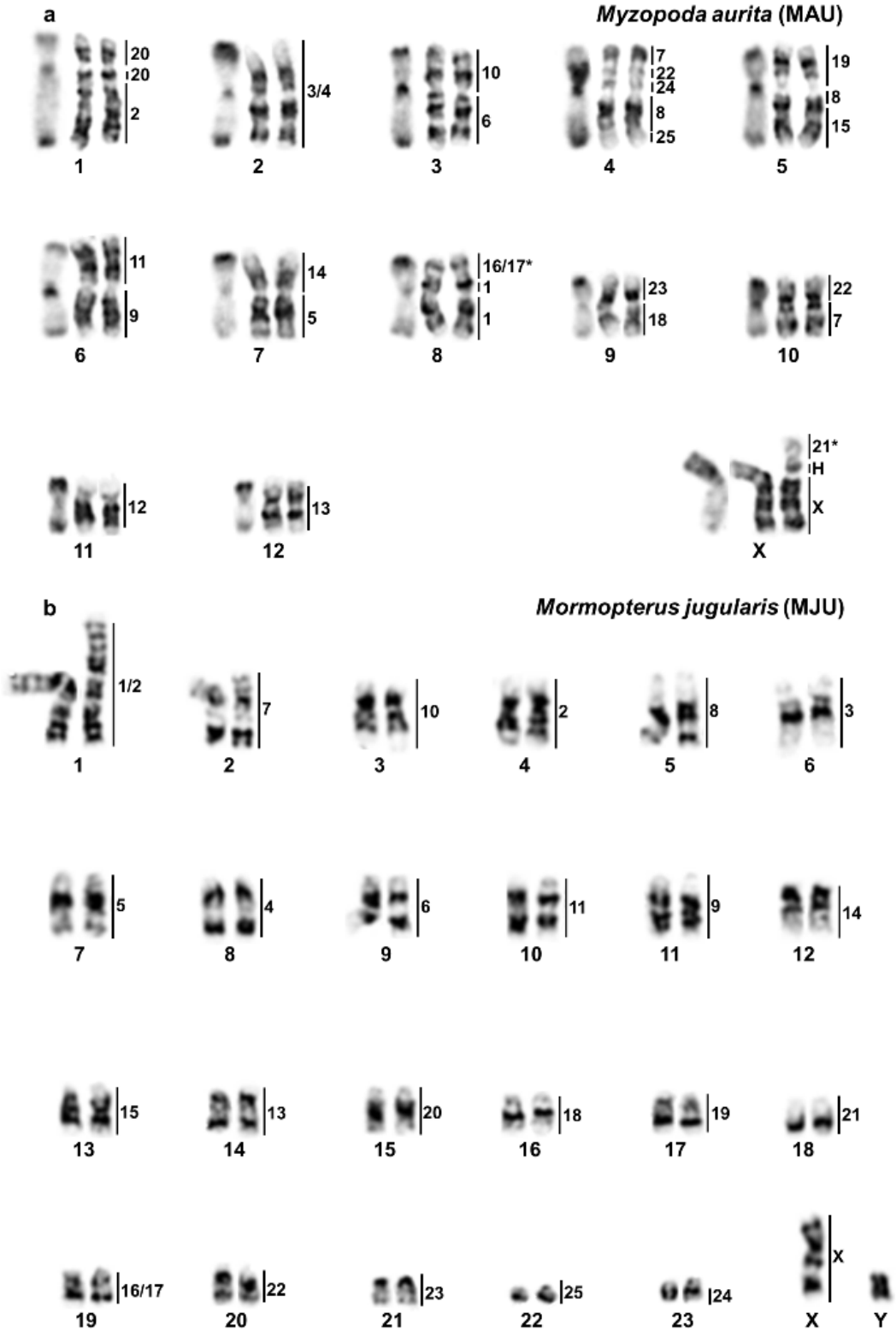

Figure 2. a The G-banded karyotype of a *Myzopoda aurita* (2n=26) with the C-banded homologue on the left side of each chromosomal pair and **b** *Mormopterus jugularis* according to Volleth et al. (2002). Chromosome numbers are given *below* each chromosomal pair. The *vertical lines* indicate chromosome painting results obtained using *Myotis myotis* probes, and the numbers *adjacent to the lines* represent *M. myotis* probes. Further painting analyses using human derived probes are required to confirm the precise positioning of MMY16/17 and 21.

In contrast, the 21 *M. myotis* probes (including the X) were retained on 24 chromosomes in *M. jugularis* (Table 4, Fig. 2b). Twenty-one *M. myotis* probes were retained on single intact chromosomes in the genome of *M. griveaudi*, highlighting 23 regions of homology between *M. myotis* and *M. griveaudi* (Table 4, Fig. 3a). Furthermore, all 21 *M. myotis* probes and the X chromosome were retained on single intact chromosomes in the genome of *M. goudoti*, corresponding to 22 regions of homology between the two *Myotis* species (Table 4, Fig. 3b). Thus, the hybridization patterns among karyotypes of *M. goudoti*, *M. griveaudi*, and *M. jugularis* are identical for all except three *M. myotis* probes: MMY1/2, 3/4, and 5/6 were retained on individual chromosomes in *M. goudoti* (2n=44); MMY1/2 and MMY5/6 (but not MMY3/4) were retained on individual chromosomes in *M. griveaudi* (2n=46); and MMY1/2 (but not MMY3/4 or MMY5/6) is retained on a single chromosome in *M. jugularis* (2n=48). The conserved HSA syntenies homologous to *M. myotis* chromosomes, established by Volleth et al. (2002), are provided in Table 4. The following HSA syntenies or evolutionary conserved units (ECUs), considered to be synapomorphies supporting chiropteran monophyly sensu Volleth et al. (2002), were present in the genomes of *Myzopoda*, *Mormopterus*, *Miniopterus*, and *Myotis*: 1a/6b homologous to MAU2p, MJU6, MGR8, MGO3/4p; 4a/10b corresponding to MAU1q, MJU1q, MGR1q, MGO1/2q; 4b/8c/19b presumed to be homologous to chromosomal segments located on MAU10p, MJU2, MGR4, MGO7; 11b/22b/12b found on MAU9p, MJU21, MGR20, MGO23; 13/8b/4c corresponded to MAU7q, MJU7, MGR2p, MGO5/6p; 18/20 bore homology to MAU3p, MJU3, MGR9, MGO10. The HSA 5a/7b/16b synteny, homologous to MMY8, was disrupted within the *M. aurita* genome.

Genome-wide chromosomal correspondence between *Myzopoda* and the Molossidae, Miniopteridae, and Vespertilionidae

By integrating our chromosome painting and GTG-banding data, we established a genome-wide comparative map detailing the level of genome conservation among the four species investigated in this study (Fig. 4). Chromosomes of each species were arranged according to the chromosome complement of *M. aurita* in order to contrast the highly rearranged biarmed karyotype of *Myzopoda* with those of other Malagasy chiropteran families with high diploid numbers (2n=44–48). Seven of the twelve bi-armed chromosomes of *Myzopoda* corresponded to two autosomal arms in *Mormopterus*, *Miniopterus*, and *Myotis* (Fig. 4). Most of the bi-armed chromosomes of *M. aurita* were the product of Rb fusions of two homologous acrocentric autosomes in *Mormopterus*, *Miniopterus*, and *Myotis*, as was the case for MAU6 and MAU9.

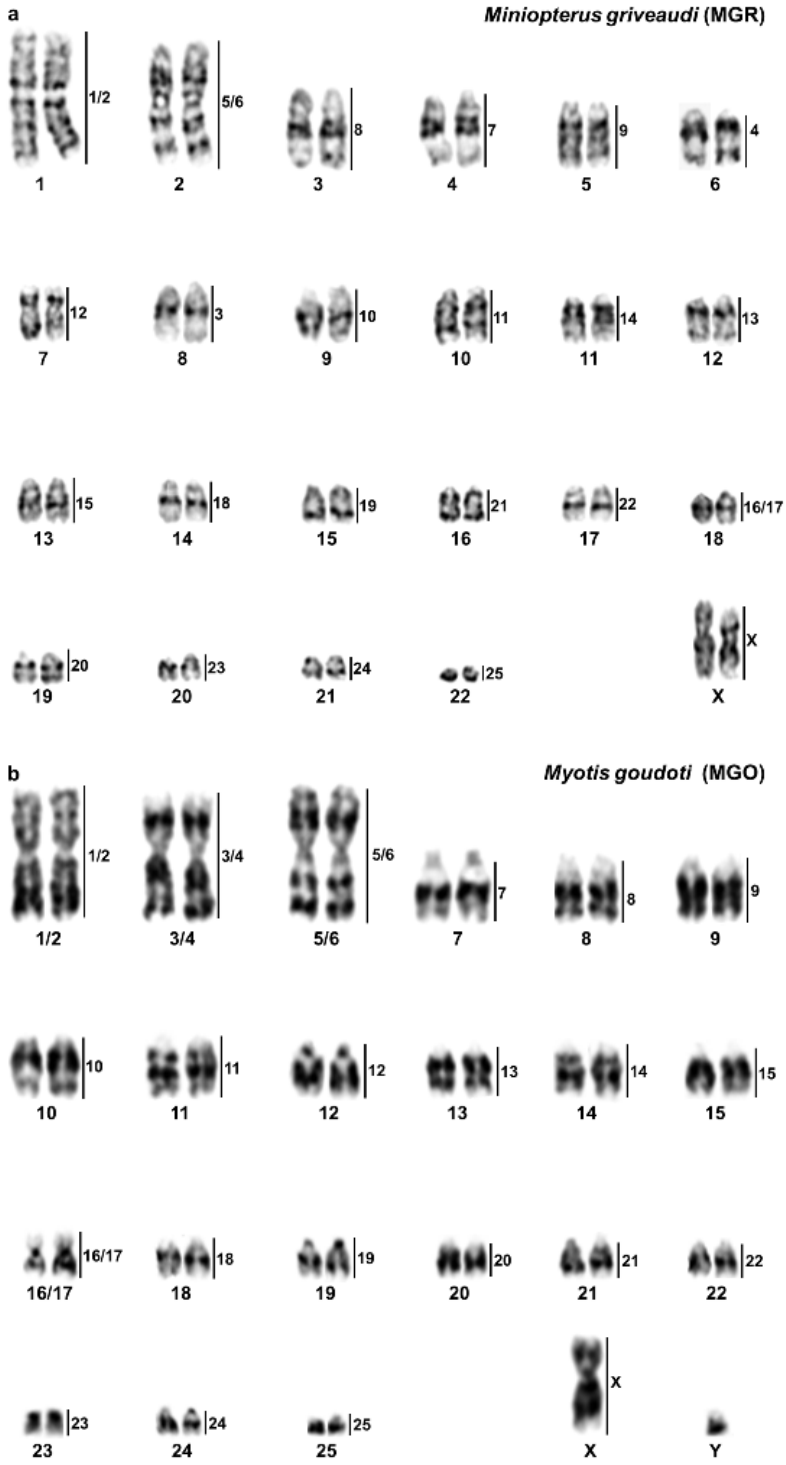

Figure 3. G-banded karyotypes of **a** *Miniopterus griveaudi* with chromosomes arranged according to the scheme proposed by Bickham (1979a) and **b** *Myotis goudoti* with chromosomes arranged from largest to smallest according to Ao *et al*. (2006). *Vertical lines* indicate the extent of hybridization sites produced by *Myotis myotis* painting probes, which are represented by numbers *adjacent* to the lines.[2]

[2] Repeat painting experiments utilising *Miniopterus griveaudi* and *Myotis goudoti* cell-cultured material revealed some chromosomal misidentifications and/or mispairing of Richards et al. (2010) that were originally based upon bone-marrow chromosomal harvests. These misidentifications have been corrected in Fig. 3 a, b; the changes do not affect the outcomes of the study.

Monobrachial homologies included: MAU1 homologous to MJU1q and 15, MGR1q and 19, MGO1/2q and 20; MAU3 homologous to MGR2q and 9, MGO5/6q and 10; and MAU7 homologous to MGR2p and 11, MGO 5/6p and 14. Two whole chromosomes were shared in toto between *M. aurita* and the other genera: MAU11 homologous to MJU4, MGR7, and MGO12 and MAU12 homologous to MJU14, MGR12, and MGO13. In addition, MAU2 was homologous to MGO3/4, a bi-armed chromosome not present in *Mormopterus* and *Miniopterus*. Chromosome painting revealed that MAU4 corresponds to the fusion of five separate autosomes/autosomal segments of *Mormopterus*, *Miniopterus*, and *Myotis*, whereas the fusion of three autosome/autosomal segments was necessary to derive MAU5 (Fig. 4). Our GTG-banded comparative map indicates that tandem fusions could be involved in the formation of MAU4 and 5 (Fig. 4).

Table 4. Chromosomal correspondence among *Myotis myotis* (MMY), *Myzopoda aurita* (MAU), *Mormopterus jugularis (*MJU), *Miniopterus griveaudi* (MGR) and *Myotis goudoti* (MGO) as revealed by cross-species chromosome painting with MMY whole-chromosome painting probes. Underlined syntenies represent bat-specific segment combinations (Volleth et al. 2002, 2011).

MMY probe	MAU	MJU	MGR	MGO	HSA
1/2	8q and 8p (prox) + 1q	1	1	1/2	14a/15a/14b/15b + 4a/10b
3/4	2	6 + 8	8 + 6	3/4	1a/6b + 3a/21
5/6	7q + 3q	7 + 9	2	5/6	13a/4c/8b/13b + 12a/22a
7	4p (dist) + 10q	2	4	7	4b/8c/19b + 5b
8	4q (prox) + 5q (prox)	5	3	8	5a/7c/16b
9	6q	11	5	9	6a
10	3p	3	9	10	18/20
11	6p	10	10	11	2a
12	11	4	7	12	7a/7b
13	12	14	12	13	11a
14	7p	12	11	14	9
15	5q (dist)	13	13	15	2b
16/17	8p (dist)	18	18	16/17	19a/16/19a
18	9q	16	14	18	10a
19	5p	17	15	19	3b
20	1p + 1q (prox)	15	19	20	8a
21	Xp (dist)	19	16	21	17
22	4p + 10p	20	17	22	1b
23	9p	21	20	23	11b/22b/12b
24	4p (prox)	23	21	24	15c
25	4q (dist)	22	22	25	1c
X	Xp (prox) + Xq	X	X	X	X

The last column provides the homology and evolutionary conserved syntenic associations of *Homo sapiens* (HSA) chromosomal segments in Malagasy Chiroptera following Volleth et al. (2002). *p* short arm; *q* long arm; *prox* proximal portion of chromosome arm; *dist* distal portion of chromosome arm.

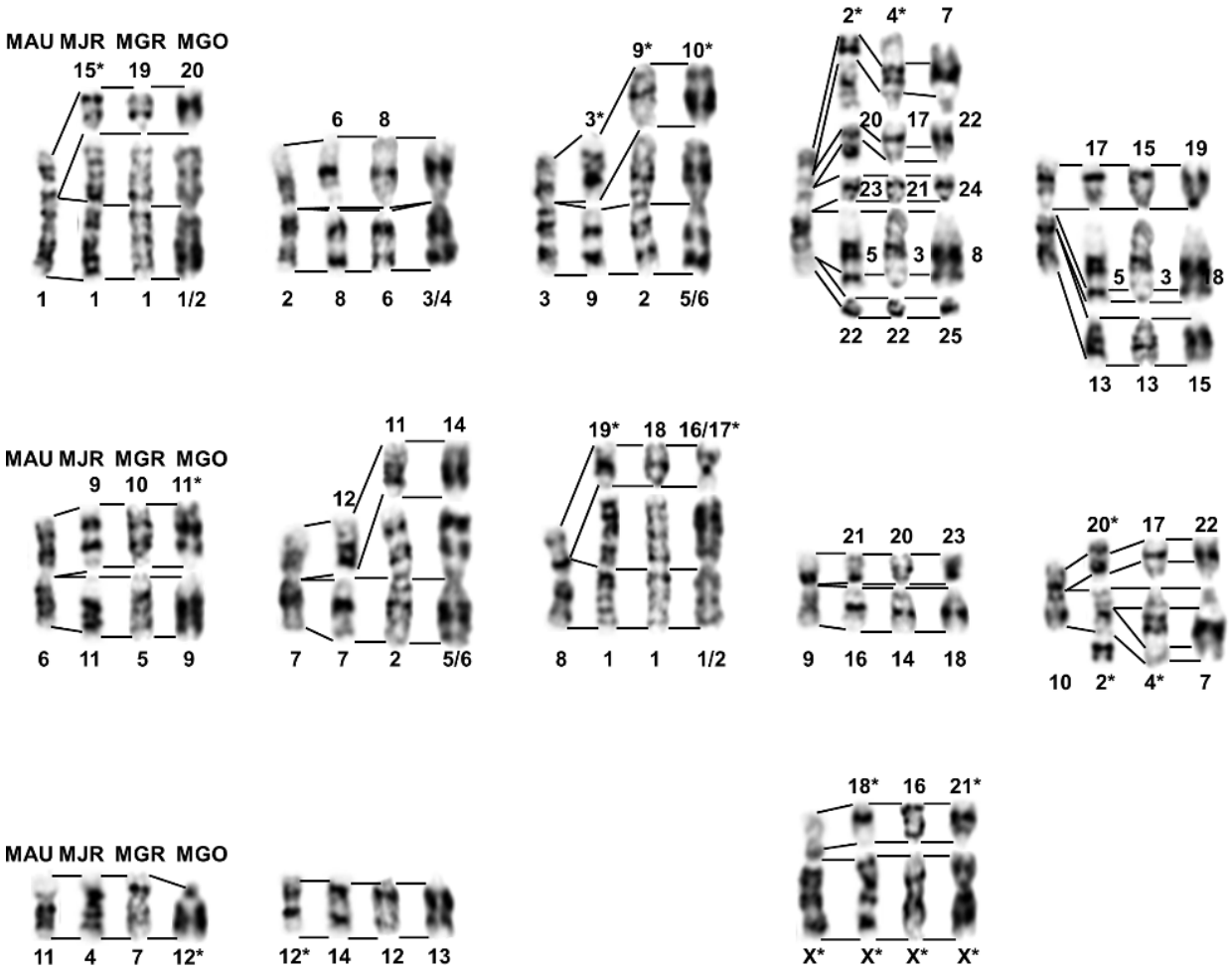

Figure 4. Genome-wide chromosomal correspondence among G-banded chromosomes of *Myzopoda aurita* (*MAU*), *Mormopterus jugularis* (*MJU*), *Miniopterus griveaudi* (*MGR*), and *Myotis goudoti* (*MGO*), with *M. aurit*a as the reference species. The homologies were directed by chromosome painting analyses using *Myotis myotis* chromosome-specific painting probes. *Asterisks* indicate areas where homology has been retained despite differential banding patterns. Further painting analyses using human derived probes are required to confirm various breakpoints and the precise positioning of MMY 16/17 and 21 in the genome of *M. aurita*

Phylogenomic analysis of chromosomal rearrangements

We investigated karyotypic evolution in *Myzopoda* relative to other chiropteran families by mapping the 79 chromosomal characters (Table 3) onto the relevant lineages of the consensus molecular phylogenetic tree (modified from Teeling et al. 2005; Miller-Butterworth et al. 2007, Fig. 5), which places *Myzopoda* within the Noctilionoidea (Fig. 5). Of the 79 chromosomal characters included in the data matrix (Table 3), 50 were autapomorphic characters. Six unique chromosomal fusion products were found in the karyotype of *M. aurita* (characters 10, 30, 40, 43, 48, and 73; Table 3). A further 18 characters were found to be possible homoplasies (Fig. 5). We retrieved a single synapomorphy (character 50, centric fusion of MMY9 and 11) uniting *Myzopoda* with *Glossophaga*, a representative of the Noctilionoidea. *Myzopoda* shared the homoplastic character 74 (fission of MMY7) with *Glossophaga*, *Taphozous*, and members of the Pteropodiformes represented in the consensus phylogenetic tree. Possible characters that may be common to *Myzopoda* and certain Pteropodiformes taxa represented in Fig. 5 included characters 6 (fusion of MMY 1 and 16/17), 28 (fusion of MMY5 and 14), 75 (fission of MMY8), and 79 (fission of MMY22). Only three syntenies were shared between *Myzopoda* and members of the Vespertilioniformes: character 12 (MMY3/4, shared with *Myotis*), character 50 (fusion of MMY9 and 11, shared with *Glossophaga*), and 67 (fusion of MMY 18 and 23, shared with *Taphozous*).

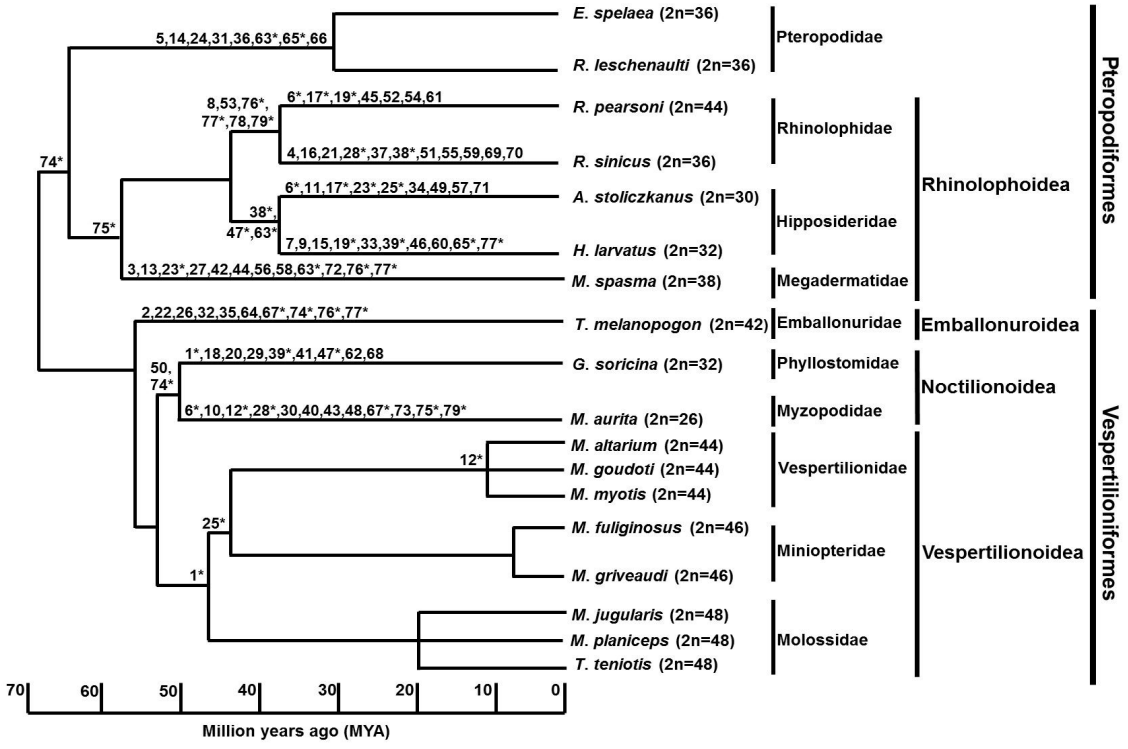

Figure 5. Karyotypic relationships and genome phylogeny of ten chiropteran families. Seventy-nine chromosomal rearrangements were mapped *a posteriori* on to a consensus molecular phylogenetic tree modified from Teeling *et al*. (2005) and Miller-Butterworth *et al*. (2007). *Numbers on branches* refer to chromosomal characters described in Table 3 and *asterisks* indicate homoplasious characters

DISCUSSION

We presented data on genome-wide chromosomal correspondence between *M. myotis* and representative species of four bat families from Madagascar, including the endemic Myzopodidae. We used genome-wide comparative maps of the species *M. aurita*, *M. jugularis*, *M. griveaudi*, and *M. goudoti* to illustrate the chromosomal rearrangements that lead to the karyotypic differentiation of the four bat families occurring on Madagascar. By comparing our maps with published comparative chromosome maps of other bat species (Volleth et al. 2002; Ao et al. 2007; Mao et al. 2007, 2008), we demonstrated karyotypic conservatism present within the genera, *Miniopterus*, *Mormopterus*, and *Myotis* such that Malagasy species were near identical to congeners from other continents. We also identified several evolutionary important characters associated with the karyotype evolution of *M. aurita*.

Karyotypic conservatism within the genera *Mormopterus*, *Miniopterus*, and *Myotis*

Comparison of our results with published data revealed a high degree of karyotypic conservatism within and among three genera of bats occurring on Madagascar: *Mormopterus*, *Miniopterus*, and *Myotis*. For example, the GTG-banding patterns of chromosomes of *M. jugularis* are near identical to those of the Australian species *M. planiceps* Volleth et al. (2002). Both species do not display the metacentric state of MMY6 (homologous to MJU9) as found in *Tadarida teniotis* (Mao et al. 2008). Karyotype conservatism appears to be characteristic of the Molossidae, as 65 of the 99 karyologically examined members of this family display a karyotype of 2n=48 and differ only in fundamental number (Sreepada et al. 2008).

The GTG-banded karyotype of *M. goudoti* was similar to that of *M. myotis* (Volleth et al. 2002) and the Asiatic *M. altarium* (Mao et al. 2007). This level of karyotypic conservatism contrasts with molecular dating estimates based on mitochondrial and nuclear DNA divergence between *M. goudoti* and *M. myotis*, which indicate that the two species last shared a common ancestor 11.39±1.5 MYA (Stadelmann et al. 2004, 2007). Karyotypic conservatism was also observed within *Miniopterus*, with the karyotype of *M. griveaudi* similar to that of *M. fuliginosus* (Ao et al. 2007). This is despite a 15.3% cyt b sequence divergence separating the two species (Goodman et al. 2009b). The same holds true for *M. aelleni* and *M. gleni,* which occur in sympatry with *M. griveaudi* within numerous cave roosts in Madagascar (Goodman et al. 2009a, b). Both *M. aelleni* and *M. gleni* carry an identical diploid and fundamental number to that of *M. griveaudi* (2n=46, NFa=50; Richards et al. unpublished), yet are distinguished from *M. griveaudi* by cyt b genetic distances of 9.9% and 10.5%, respectively (Goodman et al. 2009b).

Phylogenomic relationships between *Myzopoda* and other chiropteran families

The diploid number of 2n=26 makes *M. aurita* one of the few bat species with a diploid number lower than 30. Other species with low diploid numbers include pteropodids (*Balionycteris maculata* (2n=24), Yong and Dhaliwal 1976; *Megaerops niphanae* (2n=26), Hood et al. 1988); emballonurids (*Saccopteryx canescens* (2n=24), Hood and Baker 1986), and vespertilionids (*Lasionycteris noctivagans* (2n=20), Baker and Patton 1967; *Glauconycteris beatrix* (2n=22), Volleth and Heller 2007), with the lowest recorded diploid number belonging to *Vampyressa melissa* (2n=14, Gardner 1977). Our side-by-side GTG-band comparison indicates that the genome of *M. aurita* has been formed through 14 chromosomal and subchromosomal fusions, leading to a karyotype consisting solely of bi-armed chromosomes. Chromosome painting revealed two complex rearrangements involving fusion (centric and possibly tandem) of three or more MMY chromosomal segments, which include characters 43 (fusion of MMY7/8/22/24/25, Table 3) and 48 (fusion of MMY8/15/19, Table 3). Similar complex rearrangements/fusions have only been documented in *Megaderma spasma* (2n=38, Mao et al. 2008).

The mapped molecular tree (Fig. 5) showed that very few of the bi-armed chromosomes of *Myzopoda* were shared between species of other families. The only exceptions were MAU2 (homologous to MMY3/4), also present in *Myotis*, and MAU6 (Rb fusion of MMY9 and 11), common to *Glossophaga soricina* chromosome pair 2/18 (Volleth et al. 1999). The consensus phylogeny of Teeling et al. (2005) and Miller-Butterworth et al. (2007) shows character 50 as a possible synapomorphic feature uniting *M. aurita* with *G. soricina* (Fig. 5). Comparative GTG-banding and chromosome painting studies have revealed homologues of MMY9 + 11 in at least four other phyllostomid genera, to the exclusion of those comprising the Desmodontidae (Baker and Bass 1979; Sotero-Caio et al. 2011). Increased taxon sampling of representatives from the families Mormoopidae, Mystacinidae, Thyropteridae, Furipteridae, and Phyllostomidae in further chromosome painting studies is necessary to confirm this as a synapomorphic feature of the Noctilionoidea superfamily. Our mapping approach failed to provide further unequivocal evidence for the placement of *Myzopoda* with *Glossophaga* in the Noctilionoidea.

In common with the findings of Mao et al. (2008), our consensus phylogeny demonstrated the predominance of homoplasies/convergence in chromosomal evolution of the various bat families investigated. The disruption of MMY7 was present in all the representatives of the Pteropodiformes as well as in *Myzopoda*, other species of the infraorder Vespertilioniformes (i.e., *Taphozous melapogon* and *G. soricina*) and humans (Volleth et al. 2002). The fission state of MMY7 has been proposed for the ancestral eutherian karyotype (Robinson & Ruiz-Hererra 2008; Ruiz-Herrera et al. 2012). The disruption of MMY8 was considered to be confined to the Pteropodiformes and was previously suggested by Volleth et al. (2002) and Ao et al. (2007) to represent a synapomorphy uniting the Rhinolophidae and Hipposideridae (Rhinolophoidea). Mao et al. (2008) considered it a homoplastic character, as it has been detected in both the Megadermatidae and humans, and now in *Myzopoda* (this study). More recent preliminary

painting studies have also revealed two MMY8 homologous elements within the genomes of *Nycteris* and *Emballonura* (Volleth 2013). The chromosomal breakpoints of the homologues in these primitive Vespertilioniformes taxa differ from those found in Rhinolophoidea (Volleth 2013). Additional painting studies employing human paints will be able to verify the chromosomal breakpoints in *Myzopoda*. Other characters shared between *Myzopoda* and the Pteropodiformes included character 6, character 28, and character 79. That chromosomal evolution in *Myzopoda* has, in part, been characterized by the retention of plesiomorphic characters (e.g., character 74) lends support to the consideration of the family as one of the more primitive members of the Vespertilioniformes (Van Den Bussche and Hoofer 2001; Hoofer et al. 2003; Van Den Bussche et al. 2003).

Karyotypic evolution within Noctilionoidea and Vespertilionoidea

Comparison of *M. myotis* probe-based chromosome painting results from the four Malagasy bat species with previously published comparative chromosome maps of representatives of the Noctilionoidea (Volleth et al. 1999) and the Vespertilionoidea (Volleth et al. 2002; Ao et al. 2006; Mao et al. 2008; this study) provided further insights into chromosomal evolution within these two superfamilies.

The phylogeny of Miller-Butterworth et al. (2007) identified *Myzopoda* as a basal member of the Noctilionoidea and *Natalus* (2n=36, Baker and Jordan 1970, Kerridge and Baker 1978) as the most ancestral genus of the Vespertilionoidea. Molecular sequence-based dating placed the divergence between the two superfamilies at approximately 55 MYA (Miller-Butterworth et al. 2007). This divergence was typically associated with the retention of character 74 (fission of MMY7) within the Noctilionoidea lineage. Although chromosome painting revealed that segments of MMY7 were contained within MAU4 and MAU10, cross-species painting using human chromosome probes is necessary to determine more precisely which chromosomal segments are associated with each *M. aurita* autosome. Two separate inserts of MMY7 may also be found on chromosomes 6/7 and 1 in *G. soricina* (Volleth et al. 1999, Table 4 in Mao et al. 2008). GTG-banding studies of several phyllostomid bats, the most derived family within the Noctilionoidea, revealed that chromosomes 6/7 and 1 are also present in the karyotypes of the genera *Brachyphylla*, *Erophylla*, *Monophyllus*, and *Phyllonycteris* (Baker and Bass 1979). GTG-banded chromosomes/chromosomal arms homologous to GSO6/7 and 1 were also identified in the karyotype of *Noctilio albiventris* (Patton and Baker 1978), a representative of the Noctilionidae. This suggests that the fission state of MMY7 may be a feature in the karyotypes of genera/families belonging to Noctilionoidea.

The fusion state of MMY7 is considered the single unambiguous synapomorphy uniting the Natalidae, Molossidae, Miniopteridae, and Vespertilionidae (superfamily Vespertilionoidea) (see Volleth et al. 2002; Mao et al. 2008, this study). This autosome is in a metacentric state in *M. jugularis* and is acrocentric in *M. griveaudi* and *M. goudoti* (see Fig. 4). That *Mormopterus* is

an older lineage in the Vespertilionoidea, having diverged from *Miniopterus* and *Myotis* 54–43 MYA (Miller-Butterworth et al. 2007), and bearing a karyotype similar to that of *Natalus* (Volleth et al. 2002), suggests that the ancestral condition of MMY7 in this superfamily was bi-armed. The divergence of the Miniopteridae from the Molossidae was accompanied by the Rb fusion of MJU7 and 9 (character 25, Table 3), producing the large metacentric chromosomal pair in *M. griveaudi* (MGR2; homologous to MMY5/6), and two pericentric inversions involving MGR4 and 9 (see also Ao et al. 2006). The lineages bearing *Myotis* and *Miniopterus* split between 49 and 38 MYA (Miller-Butterworth et al. 2007), with *Myotis* differentiated from *Miniopterus* by the centric fusion of MGR6 and 8 (leading to the metacentric MMY3/4 which corresponds to MGO3/4), and a pericentric inversion on MGO12 (homologous to MMY12).

X-autosome translocation

In addition to autosome fusions, *Myotis* painting probes detected an autosome-sex chromosome translocation involving MMY X, which corresponds to MAU Xq and possibly MMY21 (corresponding to the distal portion of the short arms of the X chromosome in *M. aurita*). Further painting studies with HSA paints are necessary to confirm the precise positioning of MMY21 and 16/17, which flow-sort together. While X-autosome translocations are common in New World phyllostomid genera such as *Artibeus*, *Carollia* and *Chaeroniscus* (Hsu et al. 1968; Tucker and Bickham 1989), only two cases have been reported in Old World species belonging to the Vespertilionidae. These include *Glischropus tylopus* (2n=30/31, Volleth and Yong 1987) from Malaysia and *Glauconycteris beatrix* (2n=22/23, Volleth and Heller 2007) from central Africa. Hence, the X-A translocation in *Myzopoda* represents the third known occurrence in an Old World species. However, as only two females were investigated, we can only predict males of this species to have a diploid number of 2n=27. A C-positive intercalary heterochromatic block (IHB) was detected above the centromere in the short arm of the X-A translocated chromosome of *M. aurita* (Fig. 2a). Such IHBs are also present in X chromosomes of *G. tylopus* (Volleth and Yong 1987) and *Carollia brevicauda* (Parish et al. 2002).

X-A translocations pose two problems in effective meiotic pairing (Dobigny et al. 2004b). The first results from the difference in replication times of the autosomal (early-replication) and gonosomal (late-replication) segments (Sharp et al. 2002). Second, X-inactivation (Lyon 1968), important in the maintenance of balanced X-linked gene expression between males and females, cannot be transferred to the translocated autosome (Dobigny et al. 2004b). Silencing of autosomal genes could result in zygotic abnormalities and subsequent death (Sharp et al. 2002). Studies into the composition of such IHBs in X-A translocated chromosomes (e.g., Parish et al. 2002, Dobigny et al. 2004b) revealed them to be composed largely of 5S and 28S rDNA clusters and highly-amplified telomeric repeats. The heterochromatic block separates the early-replicating autosomal and late-replicating gonosomal segments, preventing the transmission of the X-inactivation signal from the sex genes to those of the translocated

autosome (Volleth and Yong 1987, Sharp et al. 2002, Dobigny et al. 2004b). Further, fluorescent in situ hybridization studies using telomeric and ribosomal probes may confirm the presence of telomeric repeats and rDNA sequences within the IHB of the X chromosome in *M. aurita*.

CONCLUSIONS

In this study, we used comparative chromosome painting to investigate karyotypic evolution of four genera of bats occurring in the Malagasy region. By doing so, we increased the taxon sampling in chromosome painting studies of the Chiroptera to 10 of the 20 recognized chiropteran families. Chromosomal evolution in bats is largely driven by Rb fusions (Baker and Bickham 1980, Ao et al. 2006, 2007, Mao et al. 2007, 2008) and bats from the Malagasy region are not the exception to this rule as they appear to exhibit this default rearrangement. In this study, we identified 14 Robertsonian fusions separating *M. aurita* from three species (all of which also differ by several fusions). While the majority of these fusions and the syntenic associations that were described in this study are largely autapomorphic or homoplastic (see also Mao et al. 2008), we are able to some extent demonstrate the evolutionary association of Malagasy bats to other extralimital species. For instance, our data indicates that the placement of *Myzopoda* within the Noctilionoidea can be supported by a single synapomorphy, as found by other datasets (Teeling et al. 2005, Miller-Butterworth et al. 2007). Chromosome painting data from representatives of the families Furipteridae, Mormoopidae, Mystacinidae, Noctilionidae, and Thyropteridae are required to fully understand the phylogenomic relationships within the Noctilionoidea. Further, we also describe an X-autosome translocation which has previously only been described in the families Phyllostomidae and Vespertilionidae. Additional cytogenetic work on *Myzopoda* using human derived probes would confirm and/or provide further insights into the segmental chromosomal associations and tandem fusion events proposed to be involved in the genome evolution of this remarkable chiropteran family.

ACKNOWLEDGMENTS

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CHAPTER THREE

FAMILY AND GENUS LEVEL INVESTIGATIONS

KARYOTYPIC EVOLUTION IN MALAGASY FLYING FOXES (PTEROPODIDAE, CHIROPTERA) AND THEIR RHINOLOPHOID RELATIVES AS DETERMINED BY COMPARATIVE CHROMOSOME PAINTING

L.R. Richards[1,2], R.V. Rambau[3], S.M. Goodman[2,4,5], P.J. Taylor[2,6], M.C. Schoeman[2], J.M. Lamb[2]

[1] Durban Natural Science Museum, P O Box 4085, Durban, 4000, South Africa;
[2] School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Durban, 4001, South Africa;
[3] Evolutionary Genomics Group, Department of Botany and Zoology, University of Stellenbosch, Stellenbosch, South Africa, Private Bag X1, Matieland, 7602, South Africa;
[4] Field Museum of Natural History, Science and Education, 1400 S Lake Shore Drive, Chicago, IL 60605 USA;
[5] Association Vahatra, BP 3972, Antananarivo 101, Madagascar;
[6] School of Environmental Sciences, University of Venda, Private Bag X5050, Thohoyandou, 0950;

* Author for Correspondence
LR Richards; Tel: +27 31 322 4215; Fax: +27 31 311 2242; E-mail: Leigh.Richards@durban.gov.za

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Keywords: Chiroptera, Chromosome painting, Hipposideridae, Madagascar, *Myotis myotis*, Pteropodiformes

ABSTRACT

Pteropodidae and Hipposideridae are two of the eight chiropteran families that occur on Madagascar. Despite major advancements in the systematic study of the island's bat fauna, few karyotypic data exist for Malagasy species. We utilised G- and C-banding in combination with chromosome painting employing *Myotis myotis* probes to establish genome-wide homology among Malagasy species belonging to the families Pteropodidae (*Pteropus rufus*, 2n = 38; *Rousettus madagascariensis*, 2n = 36), Hipposideridae (*Hipposideros commersoni*, 2n = 52), and a single African representative of the Rhinolophidae (*Rhinolophus clivosus*, 2n = 58). Painting probes of *M. myotis* detected 26, 28, 28, and 29 regions of homology in *R. madagascariensis*, *P. rufus*, *H. commersoni*, and *R. clivosus*, respectively. Translocations, pericentric inversions, and heterochromatin addition were responsible for karyotypic differences

amongst the Malagasy pteropodids and painting of *P. rufus* revealed a cryptic pericentric inversion on PRU 4. Chromosomal characters suggest a close alliance between *Rousettus* and *Pteropus. Hipposideros commersoni* shared several chromosomal characters with extralimital congeners, but did not exhibit the two chromosomal synapomorphies proposed for Hipposideridae. This study provides further insight into the chromosomal rearrangements that have been proposed for the ancestral karyotypes of pteropodid and rhinolophoid bats.

INTRODUCTION

Madagascar boasts a unique chiropteran fauna that includes two of the six families within the suborder Pteropodiformes: Pteropodidae (Old World fruit bats or flying foxes) and Hipposideridae (Old World leaf-nosed bats). Given the timeline between the first appearance of bats in the fossil record dating from the early Eocene and the separation of Madagascar from Gondwana [Storey et al., 1995; Simmons et al., 2008], the island was colonized on multiple occasions via overwater dispersal, which in turn gave rise to a mainly endemic taxa [Goodman, 2011]. Hypotheses concerning the evolutionary history of certain Malagasy pteropodid and hipposiderid taxa remain ambiguous, as phylogenies are not fully resolved at the generic and specific levels for both families worldwide.

Three endemic flying fox species classified to two subfamilies [sensu Bergmans, 1997] occur on Madagascar: *Eidolon dupreanum*, *Rousettus madagascariensis* (subfamily Rousettinae), and *Pteropus rufus* (subfamily Pteropodinae). Molecular studies have disputed the traditional classification of the Rousettinae and Pteropodinae and have posed novel hypotheses concerning the evolutionary relationships among pteropodids. For example, DNA based analyses are consistent in recognizing *Rousettus* and the Indomalayan *Eonycteris* as sister taxa, to the exclusion of *Eidolon* and other rousettine genera [Giannini and Simmons, 2003, 2005; Almeida et al., 2011]. *Rousettus madagascariensis* is considered the most derived species within this abridged rousettine clade [Almeida et al, 2011] and the sister species to *R. obliviosus* of the nearby Comoros Islands [Goodman et al., 2010b]. Pteropodinae as defined by Bergmans [1997] is polyphyletic as it includes at least two clades that have evolved independently from each other [Almeida et al., 2011]. *Pteropus*, the most speciose pteropodine genus, is closely allied with the Australasian *Acerodon* [Giannini and Simmons, 2005; Almeida et al., 2011]. *Pteropus* is suggested to have dispersed to Madagascar via Aldabra from Australasia / Indomalaysia, with the Malagasy species representing a more recently evolved taxon [O'Brien et al., 2009; Chan et al., 2011]. *Eidolon*, an Afrotropical endemic, does not bear close evolutionary affinities to any other genus [Almeida et al., 2011].

The Malagasy hipposiderid fauna comprises four endemic species belonging to the genera *Hipposideros*, *Paratriaenops*, and *Triaenops*. *Hipposideros commersoni* is the sole representative of the genus in Madagascar. Intergeneric relationships amongst Hipposideridae

remain unresolved as molecular phylogenies are either poorly-sampled or are incongruent in describing basal relationships amongst the genera [e.g. Jones et al., 2002; Wang et al., 2003; Li et al., 2007]. Most of the debate arises from the positioning of the genera *Aselliscus* and *Hipposideros* within the family tree. Phylogenies derived from morphological data placed *Aselliscus* at the root of the hipposiderid tree [Hand & Kirsch 1998, 2003], whilst gene-based investigations position the genus within *Hipposideros* (Wang *et al*. 2003), or at the terminal branches of the hipposiderid tree [Li *et al*. 2007]. The most recent and comprehensive molecular investigation primarily based on Afrotropical taxa, showed *Hipposideros* as the most basal lineage in clade containing the genera *Asellia*, *Coelops* and *Aselliscus* [Benda & Vallo 2009]. Furthermore, molecular studies suggest that the large Afrotropical endemics, *Hipposideros gigas* and *H. vittatus* represent some of the most ancestral forms within the genus [Eick et al., 2005; Vallo et al., 2008; Benda and Vallo, 2009; Monadjem et al., 2013]. It has yet to be determined whether the other large Afrotropical hipposiderids, including the Malagasy *H. commersoni*, exhibit similar traits. Currently, there are no available molecular phylogenies with the comprehensive species-level coverage of hipposiderids necessary for the fine-scale resolution of phylogenetic relationships amongst this diverse group [Murray et al. 2011].

Karyotypic evolution may advance at a slower pace than nucleotide evolution [Murphy *et al*., 2004]; thus, chromosomal rearrangements are rare genomic markers capable of retracing common ancestry at different taxonomic levels [Rokas and Holland, 2000]. Chromosomal banding and chromosome painting studies of Chiroptera, have demonstrated the occurrence of Robertsonian (Rb) rearrangements, inversions and heterochromatin addition in genomic restructuring amongst pteropodids and hipposiderids [Haiduk et al., 1981; Ao et al., 2007; Mao et al., 2008; Mao et al., 2010; Volleth et al., 2011]. Painting studies have also identified several clade-specific chromosomal characters in support of molecular hypotheses concerning evolutionary relationships amongst Pteropodidae and Hipposideridae [Ao et al., 2007; Mao et al*.,* 2008, 2010; Volleth et al., 2011]. To date, no data have been available to determine whether these plesiomorphic and/or synapomorphic characters are present within the Malagasy representatives of these families, as insular species are under-represented in chromosome painting studies.

In this study, we present G- and C-banded karyotypes of Malagasy endemic Pteropodidae and Hipposideridae species. Using chromosome painting with *Myotis myotis* as the overlay, we examine mode (/s) of chromosomal evolution among the Malagasy species relative to their congeners. Secondly, utilizing chromosomal characters identified from published chromosomal maps of extralimital taxa, we (a) infer phylogenomic relationships among Malagasy pteropodids and their rhinolophoid relatives, and (b) assess recent molecular-based hypotheses concerning evolutionary relationships among Pteropodidae and Hipposideridae. Thirdly, we tested for the presence of previously described synapomorphic characters proposed for Pteropodidae and Hipposideridae within the genomes of their Malagasy representatives. Our comparative analyses provide novel insights into the phylogenomic relationships among the

Malagasy taxa and the proposed chromosomal rearrangements that comprise the ancestral karyotypes of the Pteropodidae and Hipposideridae.

MATERIAL AND METHODS

The four species used in this study were collected from wild populations in Madagascar and South Africa (table 1). Specimens were identified using external morphological characteristics (e.g. forearm length) and/or echolocation characteristics [Monadjem et al., 2010; Goodman, 2011]. Bat capture and euthanasia were conducted according to ethical guidelines of the American Society of Mammalogists [Sikes and Gannon, 2011], and with the approval of the Animal Ethics Committee of the University of KwaZulu-Natal, Westville Campus.

Table 1. Chiropteran species investigated in this study.

Species name and abbreviation	Locality	GPS coordinates	Number and sex	2n	FN	Accession number
Rousettus madagascariensis (RMA)	Grotte d'Anjohibe, Province de Mahajanga, Madagascar	15.613611 S, 46.927500 E	1 ♂	36	66	FMNH 209106
Pteropus rufus (PRU)	Captive in Ambovondramanesy village, near Berivotra, Province de Mahajanga, Madagascar	-15.9000 S, 46.575 E	1 ♂	38	68	UADBA 43751
Pteropus rufus	Captive in Ambovondramanesy village, near Berivotra, Province de Mahajanga, Madagascar	-15.9000 S, 46.575 E	1 ♂	38	68	UADBA 43763
Hipposideros commersoni (HCO)	Grotte d'Anjohibe, Province de Mahajanga, Madagascar	-15.613611 S, 46.927500 E	1 ♂	52	60	FMNH 209110
Hipposideros commersoni	Réserve Spéciale d'Ankarana, Province d'Antsiranana, Madagascar		1 ♀	52	60	FMNH 213588
Rhinolophus clivosus (RCL)	Ferncliffe Nature Reserve, Pietermaritzburg, South Africa	-29.550000 S, 30.320000 E	1 ♀	58	60	DM 12005

DM = Durban Natural Science Museum; FMNH = Field Museum of Natural History, Chicago; UADBA = Université d'Antananarivo, Département de Biologie Animales

Specimens examined

Voucher specimens were deposited in the Durban Natural Science Museum (DM), South Africa; the Field Museum of Natural History (FMNH), Chicago, USA; or Université d'Antananarivo, Département de Biologie Animale, Antananarivo, Madagascar.

Cell culture, chromosome preparation and G- and C-banding

Metaphases were obtained from bone marrow preparations using the methods of Volleth et al. [2009] or fibroblast cell lines that were established from tail and/or wing membrane biopsies, using standard cytogenetic protocols. G-banding with trypsin was done following Seabright [1971] and C-banding using barium hydroxide according to a modified method of Sumner [1972].

Cross-species chromosome painting (Zoo-FISH)

Chromosome-specific painting probes of 21 autosomes and the X chromosome of *Myotis myotis* (MMY) were generated using DOP-PCR as previously described [Ao et al., 2006]. They remain the only set of chiropteran probes that have been painted reciprocally to human chromosomes [Volleth et al., 2011], thus providing definitive resolution of *Homo sapiens* (HSA) syntenic homologies. *Myotis* probes were labelled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche Molecular Chemicals) and hybridised to metaphases of the five species investigated in this study following procedures previously described [Richards et al., 2010]. Biotin-labelled *Myotis* probes were detected using Cy3-labelled streptavidin (1:500 dilution, Amersham) and Dig-labelled probes were detected with rabbit anti-FITC (1:500 dilution, Amersham).

Image capture and data processing

FISH images were captured using the Genus System version 3.7 (Applied Imaging Corp, Newcastle, UK) with a CCD camera mounted on an Olympus BX 60 epifluorescence microscope. Hybridization signals were assigned to specific chromosomes or chromosomal segments defined by enhanced DAPI-banding patterns.

Chromosome nomenclature

The G-banded karyotypes of *R. madagascariensis* and *Pteropus rufus* were arranged according to the scheme for *Rousettus leschenaulti* by Mao et al. [2007]. The karyotypes of *H. commersoni* and *Rhinolophus clivosus* followed the scheme of *R. ferrumequinum tragatus* by Mao et al. [2007], whereby biarmed chromosomes are numbered first. To best of our knowledge, no comprehensive banding and chromosome painting data exists for the Malagasy species.

Phylogenomic comparisons using chromosomal characters

We integrated our results with the published comparative maps of an additional seven Pteropodiformes taxa [Volleth et al., 2002; Ao et al., 2007; Mao et al., 2007, 2008]. We identified chromosomal characters based on GTG-banded comparisons and *M. myotis* homology, capable of providing an independent assessment of evolutionary relationships amongst the Pteropodidae, Hipposideridae, and other rhinolophoid species. For a more meaningful

interpretation of phylogenomic relationships among taxa, we only report characters that occur in two or more species.

RESULTS

Pteropodidae - karyotypes and Zoo-FISH

Rousettus madagascariensis has a karyotype with 2n = 36, FN = 66 (fig. 1a). The chromosome complement comprises four large metacentrics (pairs 1-4), four medium-sized submetacentrics (pairs 5-7, 12), four pairs of small metacentrics (pairs 13-16), four pairs of subtelocentrics (8-11), and the single acrocentric pair 17. A secondary constriction is present on the short arm near the centromere of pair 7. The X chromosome is a large subtelocentric and the Y is the smallest and heterochromatic. Pairs 9-11 have short arms comprised mostly of heterochromatin (fig. 1c), and all chromosomes contained heterochromatin in the pericentromeric and telomeric regions.

The karyotype of *P. rufus* (2n = 38, FN = 70; fig. 1b) is characterised by 11 pairs of meta- and submetacentrics, six pairs of subtelocentric autosomes, one pair of acrocentric chromosomes, a large subtelocentric X chromosome, and a small acrocentric Y chromosome (fig. 1d). Chromosomal pair 7 displayed a secondary constriction. C-banding analysis revealed the presence of heterochromatic short arms in pairs 11, 13, 14, and 18 (fig. 1d). Heterochromatin was present in the pericentromeric and telomeric regions of all chromosomes and intercalary heterochromatic bands were detected in four pairs of bi-armed chromosomes.

The complete suite of *M. myotis* probes successfully hybridized to both pteropodid species, resulting in 26 and 29 regions of homology detected in *R. madagascariensis* and *P. rufus*, respectively (fig. 1a,b; table 2). Four probes (1/2, 3/4, 5/6, 7) hybridized to two chromosomes in the genomes of *R. madagascariensis* and *P. rufus*. The remaining probes each hybridized to a single homologous chromosome/chromosomal arm in both pteropodid species. Eight chromosomal pairs of *R. madagascariensis* (1-6, 8, and 13) corresponded to two MMY probes whereas only seven *P. rufus* autosomal pairs (1-6, 8) were highlighted by two MMY probes.

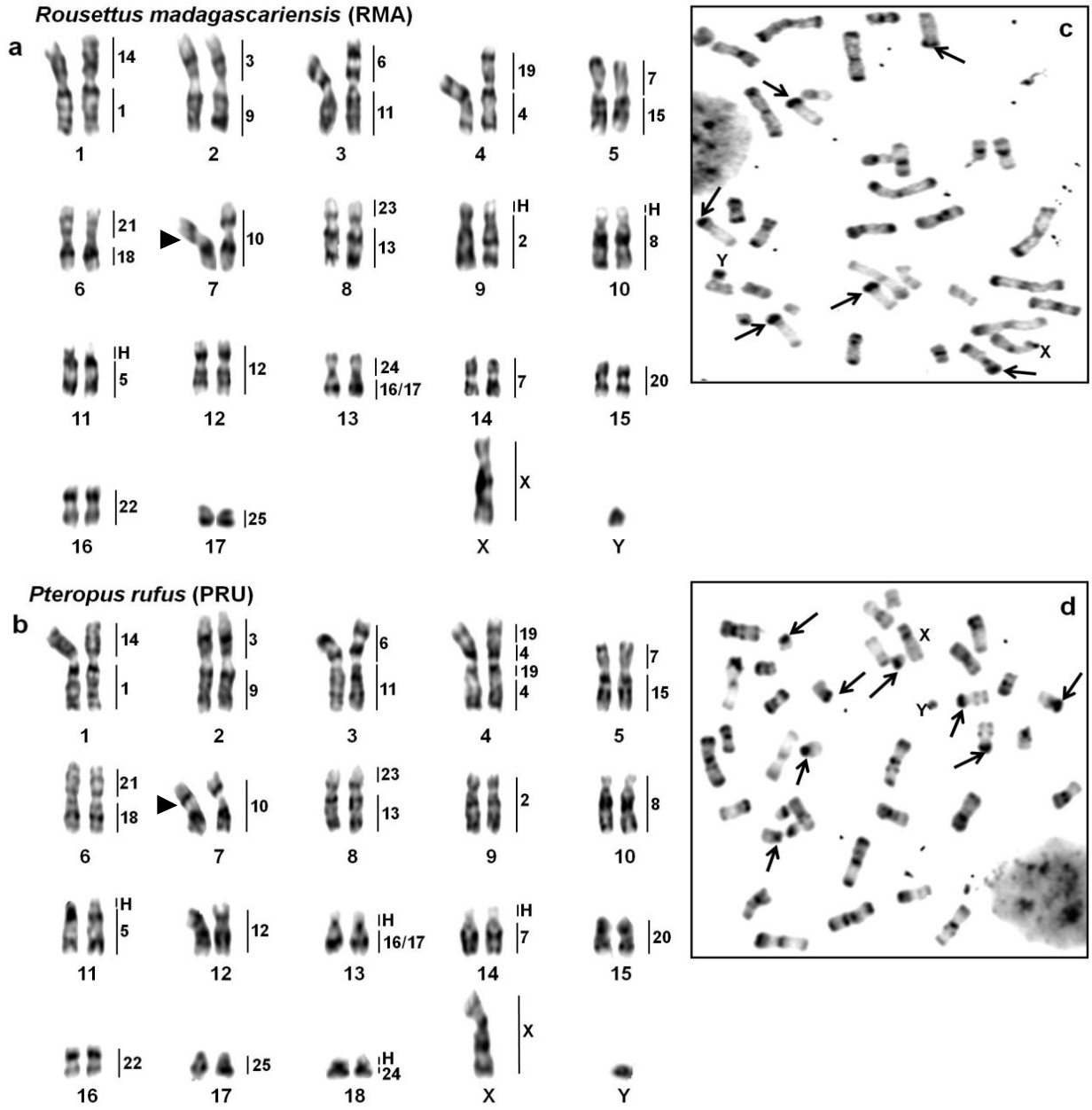

Figure 1. G-banded karyotypes of *R. madagascariensis* (RMA) (**a**) and *P. rufus* (PRU) (**b**). Chromosomal homologies to *M. myotis* (MMY) chromosomes are indicated on the right. C-banded metaphase spreads of *R. madagascariensis* (**c**) and *P. rufus* (**d**). Arrows indicate C+ heterochromatic short arms present in the Pteropodidae karyotypes. The gonosomes are indicated by X and Y.

Hipposideridae and Rhinolophidae - karyotypes and Zoo-FISH

The chromosomal complement of *H. commersoni* (2n = 52, FN = 60) comprised mostly acrocentric chromosomes with the exception of pairs 1-5 (fig. 2a). The biarmed chromosomes consist of a large submetacentric (pair 1), a medium-sized subtelocentric (pair 2), and three pairs of metacentrics (3-5). Chromosomal pair 16 displayed a secondary constriction within the pericentromeric region. The X chromosome is a large subtelocentric with large intercalary blocks of heterochromatin (fig. 2a and c). The Y chromosome is an acrocentric consisting almost entirely of heterochromatin. Heterochromatin was concentrated in autosomal centromeres, with intercalary heterochromatic bands detected in pairs 1-3 (fig. 2c).

Rhinolophus clivosus was included in this study as it bears a karyotype similar to *H. commersoni*. The karyotype of *R. clivosus* has a diploid number of 2n = 58 (FN = 60, fig. 2b), and is dominated by acrocentric chromosomes. Two small metacentric pairs (1-2) are present. A secondary constriction was located on pair 16. C-banding analysis revealed heterochromatin present in the telomeres and centromeres of all autosomes (fig. 2d). Several autosomal pairs appeared to contain large intercalary heterochromatic blocks.

Myotis autosomal probes detected 28 regions of homology in the genome of *H. commersoni* and delimited 29 homologous chromosomal segments in *R. clivosus* (fig. 3a and b; table 2), Thirteen MMY autosomes, including the X, were conserved as whole chromosomes in *H. commersoni*, whereas 14 MMY homologous whole chromosomes were identified in *R. clivosus*. Seven probes (1/2, 3/4, 5/6, 7, 8, 10, 12) were retained on two separate chromosomal pairs in *R. clivosus*.

Comparative analyses based on G- and C-band homology

Seven autosomal pairs corresponding to MMY 2, 5, 7i [see Volleth et al., 2011 for a description of MMY 7 partial chromosomal arms], 20, 22, and 25 were shared amongst the species investigated in this study (fig. 3). The banding patterns of three homologous chromosomes (MMY 20, 22, 25) were unaltered suggesting that they may represent ancestral elements of the suborder Pteropodiformes. G- and C-banding analyses revealed possible paracentric inversions on both the short and long arms of the X chromosomes. The karyotypes of the two pteropodids were similar except for the amount of heterochromatin present within the short arms of four homologous chromosomal pairs, two pericentric inversions and a possible tandem fusion in *R. madagascariensis* (fig. 3). Comparative painting analyses of the pteropodids, involving MMY probes 19 and 4, revealed a cryptic pericentric inversion in pair 4 of *P. rufus*, undetectable using G-banding patterns alone (fig. 4a). Hybridization patterns in the two pteropodid species differed due to the fusion of chromosomes homologous to MMY 16/17 + 24 in RMA13, and the retention of MMY 16/17 and 24 as separate chromosomes in *P. rufus* (fig. 4b).

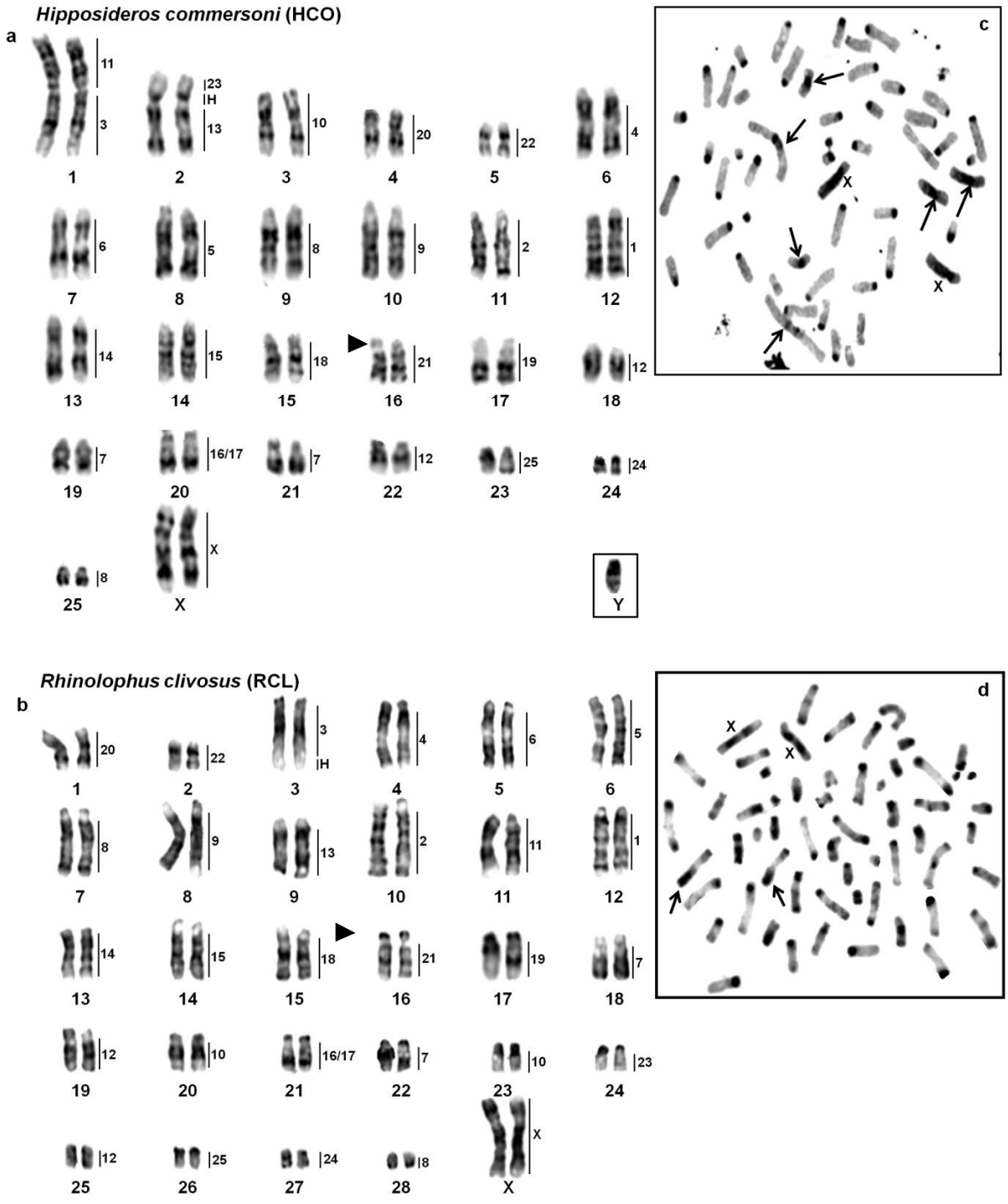

Figure 2. G-banded karyotypes of *H. commersoni* (HCO) (**a**) and *R. clivosus* (RCL) (**b**). Chromosomal homologies to *M. myotis* (MMY) chromosomes are indicated on the right. C-banded metaphase spreads of *H. commersoni* (**c**) and *R. clivosus* (**d**) are provided. Arrows indicate C+ intercalary blocks present in autosomes of the rhinolophoid bats. The gonosomes are indicated by X and Y.

Two autosomal pairs homologous to MMY 10 and the fusion of MMY 13 + 23, were retained within the genomes of the pteropodid and hipposiderid taxa. Banding patterns within the *p* arm of the chromosomes homologous to MMY 13 + 23 were conserved between *H. commersoni* and both flying fox species (fig. 3). MMY10 was conserved as a single metacentric autosome in *H. commersoni* (fig. 4c). A further two centric fusions involving MMY 3 + 11 and MMY 13 + 23 differentiated the karyotype of *H. commersoni* from *R. clivosus* (fig. 3, 4d and e). Two elements of MMY 8 and 12 were present within the hipposiderid and rhinolophid species, as well as a marker chromosome homologous to MMY 21. Marker chromosomes corresponding to MMY 10 were also identified within the karyotypes of the Malagasy pteropodids.

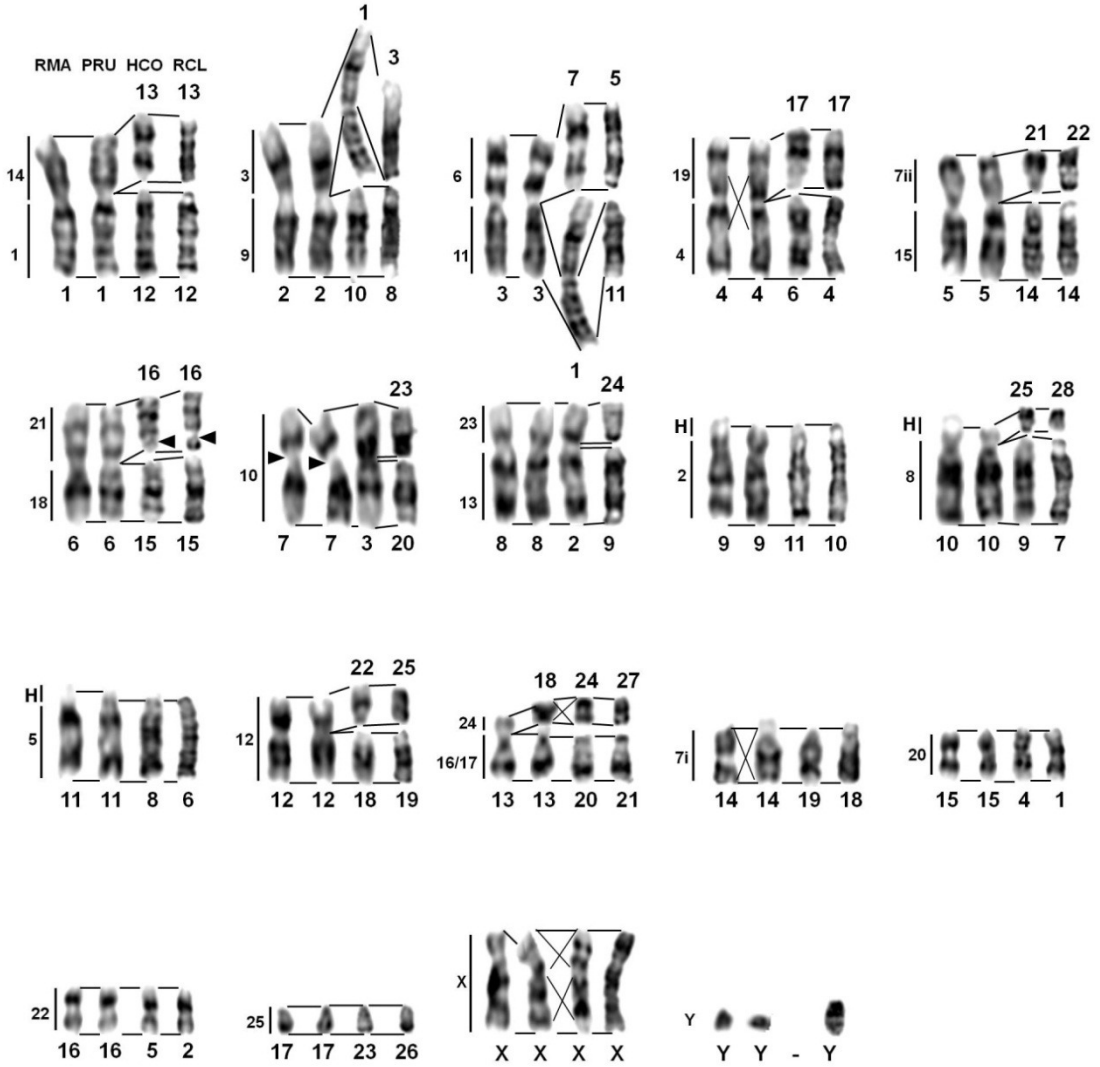

Figure 3. Genome-wide chromosomal homologies among Afrotropical pteropodid, hipposiderid and rhinolophid bats as directed by *M. myotis* chromosome painting probes and G-banding comparison. Chromosome numbers are provided below or above the chromosomes/ chromosomal segments of each species. Chromosomal homologies to *M. myotis* (MMY) chromosomes are indicated on the left. Arrowheads indicate secondary constrictions, whilst crossed lines demonstrate possible paracentric and pericentric inversions. RMA, *Rousettus madagascariensis*; PRU, *Pteropus rufus*; HCO, *Hipposideros commersoni*; RCL, *Rhinolophus clivosus*.

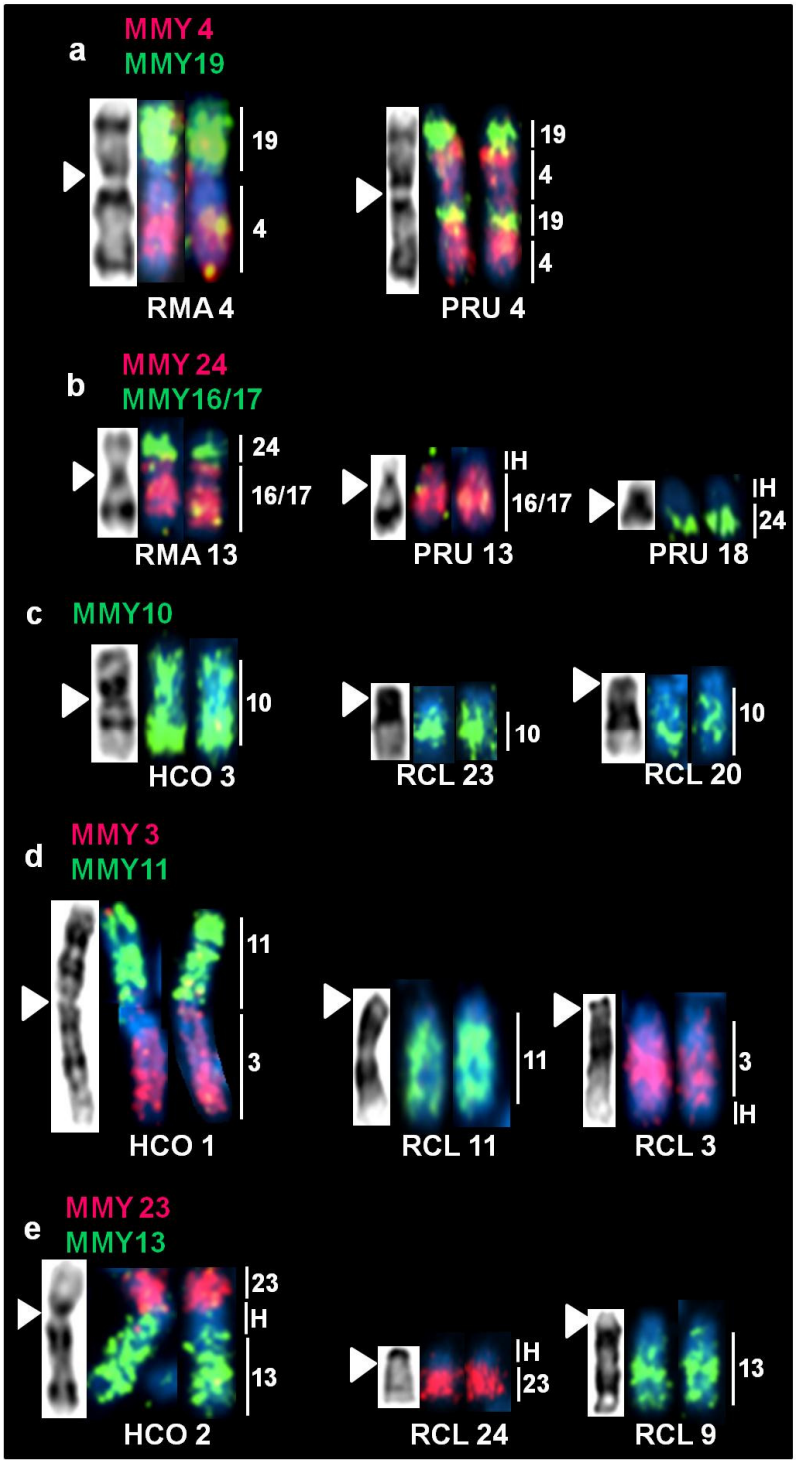

Figure 4. The results of FISH with MMY chromosomal probes onto metaphase chromosomes of *R. madagascariensis* (RMA), *P. rufus* (PRU), *H. commersoni* (HCO) and *R. clivosus* (RCL). An inversion differentiating RMA from PRU was detected using paints MMY4 and 19 **(a)**. Paints MMY24 and 16/17 revealed a fission and heterochromatic addition in PRU **(b)**. Hybridization of MMY 10 to HCO3 and RCL 20 and 23, indicating the fission of MMY10 in the genome of *R. clivosus* (**c**). MMY 11 and 3 hybridized to a single chromosomal pair in *H. commersoni* and two separate autosomes in *R. clivosus* (**d**). MMY 13 and 23 were retained on a single chromosomal pair in *H. commersoni* and as two separate chromosomes within the genome of *R. clivosus* (**e**). Arrowheads indicate the position of centromeric regions. Chromosomes were counterstained using DAPI, while MMY3, 4, 11, 16/17 and 23 were labelled with biotin and MMY 10, 11, 13, 19 and 24 were labelled with dig paints.

Phylogenomic relationships based on chromosomal characters

Our chromosome painting data of *R. madagascariensis*, *P. rufus*, *H. commersoni*, and *R. clivosus* were integrated with comparative chromosome maps of seven additional taxa. We identified 25 chromosomal characters, summarized in table 2, that were used to describe phylogenomic relationships amongst the 11 species. Widespread monobrachial homologies resulted in few shared chromosomal rearrangements across all species, apart from the fission state of MMY 7. Possible plesiomorphic characters included the fusion state of MMY 20 and 22, and the fusion product of MMY 13 + 23. The synteny of MMY 13 + 23 was conserved amongst three pteropodid genera, all three hipposiderid species, and the single species belonging to the family Megadermatidae.

Very few chromosomal characters were common across all analysed pteropodid taxa. The secondary constriction present on chromosomes or chromosomal segments homologous to MMY 10 was the only character shared among the pteropodids, including the Malagasy representatives. Six fusion characters were shared amongst *Eonycteris*, *Rousettus* and *Pteropus*. The synteny of MMY 4 + 19 was altered in *P. rufus* due to a pericentric inversion. Homologues to MMY 16/17 + 24 were present in different combinations within the genomes of the genera *Eonycteris*, *Rousettus* and in *Hipposideros larvatus*. This Rb product was not present in genomes of *P. rufus*, *H. commersoni* and *Aselliscus stoliczkanus*.

The fission state of the MMY 8 homologue and the secondary constriction on chromosomes homologous to MMY 21 were common only to the Hipposideridae and Rhinolophidae. Our comparative analyses failed to identify synapomorphic characters for the Hipposideridae. The fusion product of MMY 3 +11 represented the only chromosome limited to *Hipposideros* spp. Similarly, the fission of MMY 12 was a feature common to only the *Hipposideros* spp., and not *Aselliscus*. MMY 20 and 22 homologues and the fission of MMY 12 were also present in the karyotype of *R. clivosus*.

Table 2. Chromosomal characters shared among11 Pteropodiformes taxa from four families.

Character	Pteropodidae					Hipposideridae			Rhinolophidae		Megadermatidae
	CSP[a] 2n = 34	ESP[b] 2n = 36	RLE[c] 2n = 36	RMA[d] 2n = 36	PRU[d] 2n = 38	AST[a] 2n = 30	HLA[b] 2n = 32	HCO[d] 2n = 52	RPE[c] 2n = 44	RCL[d] 2n = 58	MSP[e] 2n = 38
Fi 7	1	1	1	1	1	1	1	1	1	1	1
Fu 22	1	1	1	1	1	0	1	1	0	1	1
SC 10	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0
Fu 20	0	1	1	1	1	0	1	1	0	1	1inv
Fu 10	0	1	1	1	1	1	1	0	0	0	0
Fu 13 + 23	0	1	1	1	1	1	1 inv	1	0	0	1 inv
Fu 16/17 + 24	0	**1***	**1***	**1***	0	0	1	0	0	0	0
Fu 1 + 14	0	**1**	**1**	**1**	**1**	0	0	0	0	0	0
Fu 3 + 9	0	**1**	**1**	**1**	**1**	0	0	0	0	0	0
Fu 4 + 19	0	**1**	**1**	**1**	**1 inv**	0	0	0	0	0	0
Fu 6 + 11	0	**1**	**1**	**1**	**1**	0	0	0	0	0	0
Fu 7 + 15	0	**1**	**1**	**1**	**1**	0	0	0	0	0	0
Fu 18 + 21	0	**1**	**1**	**1**	**1**	0	0	0	0	0	0
Fu 3 + 7ii	1	**0**	**0**	**0**	0	0	0	0	0	0	1
Fu 8ii + 11	1	0	0	0	0	0	0	0	0	0	1
Fi 12	1	0	0	0	0	0	1	1	1	1	1
SC 21	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	0
Fi 8	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**1**
Fu 3 + 11	0	0	0	0	0	0	**1**	**1**	0	0	0
Fu 7i + 19	0	0	0	0	0	1	1	0	0	0	0
Fu 8ii + 14	0	0	0	0	0	1	1	0	0	0	0
Fu 1 + 16/17	0	0	0	0	0	1	0	0	1	0	0
Fu 3 + 15	0	0	0	0	0	1	0	0	1	0	0
Fu 4 + 5	0	0	0	0	0	0	1	0	0	1	0
Fu 4 +18	0	0	0	0	0	1	0	0	0	0	1

Characters are described based on *Myotis myotis* homologies. *Cynopterus sphinx* (CSP); *Eonycteris spelaea* (ESP); *Rousettus leschenaulti* (RLE); *R. madagascariensis* (RMA); *Pteropus rufus* (PRU); *Aselliscus stoliczkanus* (AST); *Hipposideros larvatus* (HLA); *H. commersoni* (HCO); *Rhinolophus pearsoni* (RPE); *R. clivosus* (RCL); *Megaderma spasma* (MSP). Fi = fission; Fu = Robertsonian fusion; inv = inversion ; SC = secondary constriction; * = non-centric fusion. Numbers in bold = possible synapomorphies. MMY chromosomal segments as according to Volleth et al. [2011]: 7i = HSA 19/8/4 homologous segment; 7ii = HSA 5 homologous segment; 8ii = HSA 7/5 homologous segment. Cited from: [a] Ao et al. [2007], [b] Volleth et al. [2002], [c] Mao et al. [2007], [d] this study, [e] Mao et al. [2008].

DISCUSSION

Karyotypic evolution among Malagasy pteropodids

Relative to the families Hipposideridae and Rhinolophidae, the Pteropodidae have been the least studied using chromosome painting techniques. Only three species divided amongst the subfamilies Cynopterinae and Rousettinae have been examined thus far [Volleth et al., 2002; Ao et al., 2007; Mao et al., 2007]. We present the first painting analysis of a member of the Pteropodinae, *P. rufus*. To date, karyotypic data for *Pteropus* spp. are largely derived from conventionally stained karyotypes [Harada and Tsuneaki, 1980; Rickart et al., 1989; Hood et al., 1988]. Due to the inadequacy of conventional cytogenetic studies in delimiting chromosomal rearrangements, karyotypic comparisons between *Pteropus* and other pteropodid genera have remained incomplete. Despite an overall similarity in diploid numbers of *P. rufus* (2n = 38) and *R. madagascariensis* (2n = 36), our chromosome painting analyses with *M. myotis* revealed several karyotypic differences between the Malagasy species. Chromosomal rearrangements responsible for differences in diploid number and fundamental number between Malagasy pteropodids included a single non-centric fusion, two pericentric inversions, and heterochromatin polymorphisms on four homologous chromosomal pairs. Corresponding rearrangements have been implicated in the genome evolution of African pteropodids [Haiduk et al., 1981].

Phylogenomic relationships amongst Pteropodidae

Chromosomal characters based on G-banded comparisons and chromosomes painting analyses were used to assess the phylogenomic relationships amongst five pteropodid species including the Malagasy representatives studied herein. The single character common to all pteropodid species was the secondary constriction present within chromosomes/chromosomal segments homologous to MMY 10. This marker chromosome was conserved as a single element within the karyotypes of *R. madagascariensis*, *R. leschenaulti*, *P. rufus*, and *Eonycteris spelaea*. Homologues to MMY10 appear as two elements on separate biarmed chromosomes in *Cynopterus sphinx*, one of which bears a secondary constriction adjacent to the pericentromeric region [Ao et al., 2007]. Marker chromosomes have been reported from all pteropodids analysed karyotypically, with the exception of *Scotonyteris ophiodon* [Haiduk et al., 1981]. A study of ten Philippine pteropodids revealed the secondary constriction to correspond to nucleolar organizer regions (NOR) [Rickart et al., 1989]. Additional investigations using silver-staining and /or hybridization experiments with rDNA probes will be able to determine whether this is the case for Malagasy pteropodids.

Six chromosomal characters, each representing a centric fusion, were common to the genera *Rousettus*, *Pteropus,* and *Eonycteris*. Three fusion products corresponding to MMY 3 + 9 (HSA 6), 4 + 19 (HSA 3 + 21) and 13 + 23 (HSA 11) [HSA synteny based on Volleth et al., 2002, 2011], represent conserved elements within the placental ancestral karyotype [Robinson

and Ruiz-Herrera, 2008; Ruiz-Herrera et al., 2012]. Within Chiroptera, these three chromosomal features have only been reported from pteropodids [see Volleth et al., 2011], with the exception of *C. sphinx* [Ao et al., 2007]. Comparisons between the G-banded karyotypes of *R. madagascariensis* and *P. rufus* (this study), and published karyotypes of *E. spelaea* [Volleth et al., 2002] and *R. leschenaulti* [Mao et al., 2007], revealed the banding patterns of chromosomes homologous to MMY 3 + 9 and 13 + 23 were conserved across all taxa. Our study shows the MMY homology of *P. rufus* chromosomal pair 4, corresponding to MMY 4 + 19, as distinct and more similar to the MMY syntenic arrangement in HSA 3 [see Fig. 2, Volleth et al., 2011]. Experiments with human painting probes and chromosomal probes derived from species with fragmented genomes including *Eulemur macaco* (black lemur) and *Tupaia belangeri* (tree shrew) [Volleth et al., 2011], are needed to confirm the chromosomal segmental order within PRU 4 and to determine whether it is representative of the conserved arrangement in HSA 3. Additional painting studies with human probes will also confirm the position of HSA 21 on PRU4. The arrangement in chromosomal pair 4 in *R. madagascariensi*s is the same as that described for *E. spelaea* [Volleth et al., 2002] and *R. leschenaulti* [Mao et al., 2007], and is considered as a derived state [Volleth et al., 2011]. This syntenic association may therefore represent an autapomorphy of the rousettine clade as defined by molecular DNA studies [e.g. Giannini and Simmons, 2005; Almeida et al., 2011]. The non-centric fusion of homologues to MMY 16/17 + 24 appears to be characteristic of the *Eonycteris* and *Rousettus* genera and may therefore characterize an additional synapomorphic feature of this rousettine clade. The Rb fusion of MMY 16/17 + 24 within certain hipposiderid species may therefore not represent a convergence event as previously suspected [see Volleth et al., 2002; Ao et al., 2007].

This study and other published chromosomal data [Volleth et al., 2002; Mao et al., 2007] thus support the molecular hypothesis of a close association *Eonycteris* and *Rousettus* (Rousettinae). Furthermore, chromosomal data suggests a close alliance between *Pteropus* and *Rousettus* as 16 chromosomes were shared *in toto* between these two genera. The extensive chromosomal rearrangements that have occurred between *C. sphinx* (Cynopterinae) and genera belonging to other subfamilies, renders the phylogenomic relationships amongst pteropodid genera particularly difficult to discern. The secondary constriction present on chromosomes / chromosomal segments corresponding to MMY 10 represented the only possible chromosomal synapomorphic feature of pteropodids analysed thus far. Painting analyses of additional species that display intermediate steps of chromosomal evolution between *Cynopterus* and other pteropodids are needed to fully resolve the cytosystematics of fruit bats.

Phylogenomics relationships between Hipposideros commersoni *and other hipposiderids*

Karyotype analyses of hipposiderids revealed diploid numbers varying between 2n = 30-52, with most species exhibiting a biarmed karyotype of 2n = 32 [see reviews of Bogdanowicz and Owen, 1998; Sreepada et al., 1993]. *Hipposideros commersoni*, one of four large

Afrotropical hipposiderids, exhibits an atypical diploid number of 2n = 52 [*H. vittatus*, Rautenbach et al., 1993; *H. gigas*, Koubínova et al; *H. commersoni,* Volleth et al., 2011]. Our understanding of karyotypic evolution within the family remained limited as only species with 2n = 30 [*Aselliscus stoliczkanus*; Ao et al., 2007] and 2n = 32 [*H. armiger*, *H. larvatus*, *H. pomona*, *H. pratti*; Mao et al., 2010] were studied using chromosome painting techniques. Despite this limited taxon sampling, several synapomorphic features of Hipposideridae have been proposed based on findings of chromosome painting analyses using human, *Myotis* and *Aselliscus* probes [see Volleth et al., 2002; Ao et al., 2007; Mao et al. 2010]. The syntenic associations of MMY 8 + 14 (homologous to HSA 5 + 7 + 9) and MMY 7 + 19 (HSA 3 + 9 + 4), proposed synapomorphies of Hipposideridae, were not present in the genome of *H. commersoni*. Conversely, two chromosomes corresponding to the homologues of MMY 10 and 13 + 23, considered key features of the ancestral karyotype of Hipposideridae, were conserved as biarmed elements in *H. commersoni*. MMY 13 + 23, equivalent to HSA 11 and postulated to be a synapomorphic feature for Eutheria [Robinson and Ruiz-Herrera, 2008; Ruiz-Herrera et al., 2012], was also present in four of the five pteropodid species analysed thus far.

Ao et al. [2007] proposed *A. stoliczkanus* (AST) as the likely basal taxon within Hipposideridae, as this species shared plesiomorphic chromosomal characters with pteropodids, including the retention of MMY 10 and 12 as a bi-armed elements and the arrangement of MMY 23 on the *p* arm of AST 11. Until present, all *Hipposideros* spp. studied using chromosome painting displayed a different G-banding pattern in the *p* arm of chromosomes homologous to AST 11 based on one or more paracentric inversions [see Mao et al., 2010]. Our results, however, show the G-banding pattern in the p arm of HCO 2 is the same as that of AST 11 and pteropodids, considered to be the ancestral condition. Our data also indicates that *H. commersoni* shares several chromosomal features with both the Pteropodidae (e.g. fusion state of MMY 10 and MMY 13 + 23) and the Rhinolophidae and Megadermatidae (fusion state of MMY 20 and 22 and the fission of MMY 8 and 12). *Hipposideros commersoni* also displayed a secondary constriction on the MMY 21 homologue; a feature considered diagnostic for Hipposideridae and Rhinolophidae [see Volleth et al., 2002; Mao et al., 2007]. A largely acrocentric chromosomal complement has been postulated as ancestral for both the Hipposideridae [Bogdanowicz and Owen, 1998] and the Rhinolophidae [Mao et al., 2007].

These data bring into question the supposition that *A. stoliczkanus* possesses the most primitive hipposiderid chromosomal complement [Ao et al., 2007]. Benda and Vallo [2009] demonstrate that *A. stoliczkanus* occupies a terminal branch in a clade containing the genera *Asellia* and *Coelops*, representing the successive lineage to *Hipposideros*. Furthermore, molecular phylogenies indicate the 2n = 52 *Hipposideros* species are basal to other Afrotropical *Hipposideros* spp. characterised by 2n = 32 karyotypes [Eick et al., 2005; Vallo et al., 2008; Monadjem et al., 2013]. Hence, the karyotype of *H. commersoni* may in fact be more representative of the ancestral hipposiderid chromosomal complement. The inclusion of other 2n = 52 species, such as *H. gigas* and *H. vittatus*, in future painting studies of Hipposideridae

may provide further evidence that corroborate our findings. More comprehensive painting studies that include detailed comparative maps of additional hipposiderid genera, including the recently described Malagasy *Paratriaenops* are needed to provide conclusive resolution of intergeneric phylogenomic relationships amongst the family.

CONCLUSIONS

By expanding chromosome painting studies of Pteropodidae and Hipposideridae to include Malagasy endemic species, we have refined our knowledge of the phylogenomic relationships among the two families and the chromosomal characters that have played an important role in their karyotypic evolution. Our results confirm Rb rearrangements as an important mode of karyotype evolution in Chiroptera. Despite the limitation of these rearrangements in resolving interfamilial relationships amongst bats due to widespread monobrachial homologies and convergent events [Mao et al., 2008], we found these characters (chromosomal fusion and fission events) to be useful in inferring phylogenetic relationships at the generic level [see also Sotero-Caio et al., 2011]. Our study also highlighted the utility of inversions in phylogenomic studies of Pteropodiformes taxa. Repeat experiments with HSA paints and probes derived from species with fragmented karyotypes are necessary to resolve the segmental associations of certain chromosomal elements within the karyotypes of the species studied here. These include clarifying the structural composition of PRU 4 and determining whether the paracentric inversion within the MMY 2 homologous segment, suggested to be a synapomorphy for Pteropodiformes, is present within the Malagasy taxa.

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CHAPTER FOUR

SPECIES LEVEL INVESTIGATIONS

CRANIAL SIZE AND SHAPE VARIATION IN AFROTROPICAL *OTOMOPS* (MAMMALIA: CHIROPTERA: MOLOSSIDAE): TESTING SPECIES LIMITS USING A MORPHOMETRIC APPROACH

Leigh R. Richards[1,2*], Peter J. Taylor[2,3], M. Corrie Schoeman[2], Steven M. G[,4,5,] Paul A. A. G. Van Daele[6] and Jennifer M. Lamb[*]

[1]*eThekwini Libraries and Heritage, Durban Natural Science Museum, PO Box 4085, Durban, 4000, South Africa*
[2]*School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Durban, 4001, South Africa*
[3]*School of Environmental Sciences, University of Venda, Private Bag X5050, Thohoyandou, 0950,South Africa*
[4]*Field Museum of Natural History, Department of Zoology, 1400 S Lake Shore Drive, Chicago, IL 60605, USA*
[5]*Vahatra, BP 3972, Antananarivo 101, Madagascar*
[6]*Evolutionary Morphology of Vertebrates, Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium*

ADDITIONAL KEYWORDS Africa – cranial morphology – ecogeographical variables – geometric morphometrics – giant mastiff bats – holotypes – morphological evolution – taxonomy.

ABSTRACT

The taxonomy of the Old World bat genus *Otomops* (Chiroptera: Molossidae) has been the subject of considerable debate. The failure of classical morphological studies to provide consistent patterns regarding interspecific relationships within *Otomops* has limited any understanding of the evolutionary history of the genus. We used traditional and geometric morphometric approaches to establish the species limits of taxa from sub-Saharan Africa, the Arabian Peninsula, and Madagascar. Morphometric data supported the recent recognition of three distinct Afrotropical taxa: *Otomops madagascariensis* from Madagascar; *Otomops martiensseni s.s.* from southern, eastern, central, and western Africa; and an undescribed taxon from north-east Africa and the Arabian Peninsula. Analyses of craniodental measurements and landmark-based data showed significant cranial size and shape divergence between the three taxa. Cranial size and shape variation within Afro-Arabian *Otomops* were strongly influenced by altitude, seasonality of precipitation, and precipitation in the driest month. Based on morphometric patterns and molecular divergence estimates, we suggest that morphological

evolution within Afro-Arabian *Otomops* occurred in response to the fluctuating climate during the Pleistocene on the one hand, and the increasing aridity and seasonality over north-eastern Africa on the other.

INTRODUCTION

It is widely recognized that current information on the systematics and phylogenetic history of living Chiroptera is limited, despite major advances in the past decade (Volleth *et al*., 2002; Van Den Bussche & Hoofer, 2004; Eick, Jacobs & Matthee, 2005; Miller-Butterworth *et al*., 2007). Molossidae, a wide spread family in the New and Old Worlds, are no exception. Increased sampling and the application of molecular sequencing techniques have provided insights into the evolutionary history of the various genera of this family (Ratrimomanarivo *et al*., 2007, 2008; Lamb *et al*., 2008, 2011; McDonough *et al*., 2008; Taylor *et al*., 2009). Although some molossid bats are very common and have day-roost sites in synanthropic settings, others, such as species of the Old World genus *Otomops* Thomas, 1913, are rarely collected and poorly studied. The poor representation in museum collections of members of this genus, which impedes systematic studies (Kitchener, How & Maryanto, 1992), is associated with the difficulty in capturing these high-flying, large-bodied bats by conventional methods, such as mist nets and harp traps.

Simmons (2005) recognized seven species of *Otomops*, five of which are strictly Indomalayan (including Papua New Guinea): *Otomops wroughtoni* (Thomas, 1913) from India and Cambodia; *Otomops formosus* Chasen, 1939 from Java; *Otomops papuensis* Lawrence, 1948 and *Otomops secundus* Hayman, 1952 from New Guinea; and *Otomops johnstonei* Kitchener, How & Maryanto, 1992 from Indonesia. The Indomalayan species are categorized as data deficient (IUCN, 2013) because most are only known from the original type series (Kitchener *et al*., 1992). Two species are currently recognized from the Afrotropics. *Otomops madagascariensis* Dorst, 1953 is distributed in the dry regions and Central Highlands of Madagascar (Goodman, 2011). *Otomops martiensseni* (Matschie, 1897) has a disjunct distribution across sub-Saharan Africa and the south-western portion of the Arabian Peninsula. There are records attributed to this species from Central African Republic, Democratic Republic (DR) of Congo, Djibouti, Ethiopia, Eritrea, Ghana, Ivory Coast, Kenya, Rwanda, Tanzania (type locality), Zambia, Zimbabwe, and Yemen (Al-Jumaily, 1999; Lamb *et al*., 2008).

Chubb (1917) recognized the South Africa population as a separate species, *Otomops icarus* Chubb, 1917. *Otomops icarus* has also been identified from Angola (Hill & Carter, 1941) and Malawi (Ansell, 1974). Harrison (1965) demonstrated that, based on external and craniodental measurements, *O. icarus* from South Africa were smaller in size than *O. martiensseni* from Kenya, Tanzania, and Zimbabwe. The multivariate analyses of Peterson, Eger & Mitchell (1995), however, revealed that Kenyan *Otomop*s were morphologically distinct

from specimens collected from Angola, DR of Congo, Rwanda, South Africa, Uganda, and Zimbabwe. Fenton *et al*. (2002), using forearm length, confirmed the morphological separation of specimens from South Africa (*O. icarus*) and Kenya (*O. martiensseni*). Inconsistencies in the delineation of the species limits of *O. martiensseni* and *O. icarus* using morphometric data and the failure to identify species-specific morphological characters has led to several studies questioning the taxonomic validity of the latter species (Freeman,1981; Long, 1995; Taylor, 2005). Consequently, populations from South Africa, Angola, and Malawi are considered conspecific with *O. martiensseni*, and *O. icarus* is regarded as a junior synonym of *O. martiensseni* (Simmons, 2005; Monadjem *et al*., 2010).

More recent molecular-based studies using cytochrome *b* and D-loop mitochondrial DNA sequences have clarified phylogenetic and phylogeographical patterns within *Otomops* and have raised questions regarding the taxonomic status of Afrotropical members of this genus (Lamb *et al*., 2006, 2008). Molecular data provide evidence for three distinct and reciprocally-monophyletic lineages from north-east Africa and Arabia, sub-Saharan Africa (excluding north-east Africa) and Madagascar. On the basis of these molecular studies, and including the conclusions of Peterson *et al*. (1995), the Malagasy lineage was recognized as *O. madagascariensis*. The genetic lineage described from Burundi, Ivory Coast, South Africa, Tanzania, and Zimbabwe included specimens sampled from areas neighbouring the type localities of *O. martiensseni* (Magrotto Plantation, near Tanga, southeastern foothills of the East Usambara Mountains, Tanzania; Matschie, 1897) and *O. icarus* (Durban, KwaZulu-Natal, South Africa; Chubb, 1917). The north-east lineage has been considered as an undescribed taxon occurring in Kenya, Ethiopia, and Yemen (Lamb *et al*., 2008, 2011). The discordance between the morphological and genetic delineation of Afrotropical *Otomops* and the possibility of a new species from the Afrotropics has warranted a critical assessment of the morphological diversity of *Otomops*.

The present study aimed to investigate and describe inter- and intraspecific patterns of cranial size and shape variation in Afrotropical *Otomops* using traditional and geometric morphometric approaches. More specifically, we evaluated and characterized morphological divergence between geographical populations of Afrotropical *Otomops* and tested the taxonomic validity of recently described genetic lineages (Lamb *et al*., 2008). We discussed cranial morphological variation among *Otomops* in the context of functional morphology. Finally, we assessed the adaptive role of cranial size and shape variation within Afro-Arabian *Otomops*, in relation to ecogeographical factors.

MATERIAL AND METHODS

Material examined

Crania of 202 (100 males and 102 females) *Otomops* specimens from 13 museums were examined in the present study (see Supporting information, Appendix S1): BMNH –The Natural

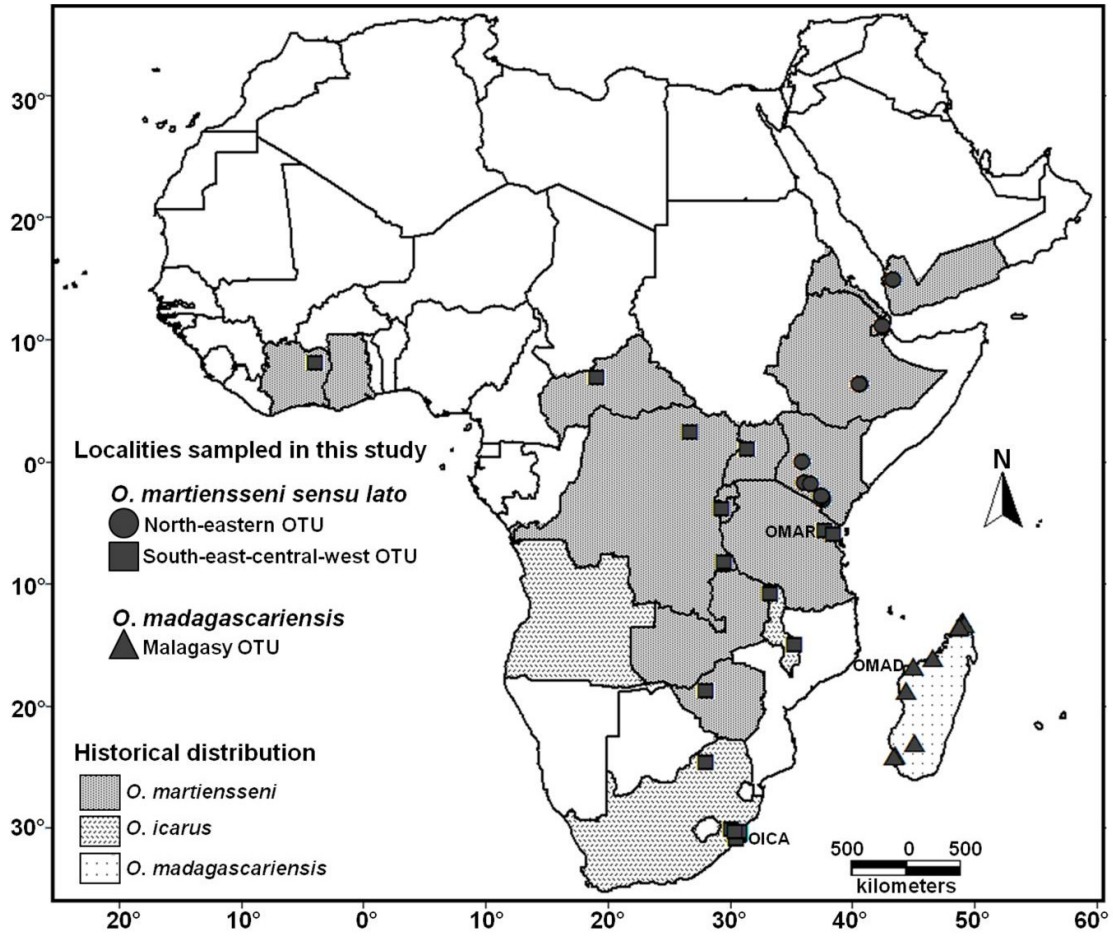

Figure 1. Map of Africa and the Arabian Peninsula showing the collecting localities for specimens included in the present study. The historical distribution of the taxa *Otomops martiensseni*, *O. icarus*, and *O. madagascariensis* are shown. Type localities of *O. martiensseni* (OMAR), *O. icarus* (OICA), and *O. madagascariensis* (OMAD) are indicated on the map.

History Museum (formerly British Museum of Natural History), London, United Kingdom; DM – Durban Natural Science Museum, Durban, South Africa; FMNH – Field Museum of Natural History, Chicago, IL, USA; HZM – Harrison Zoological Institute, Kent, United Kingdom; MNHN – Muséum National d'Histoire Naturelle, Paris, France; MRAC – Musée Royale d'Afrique Centrale, Tervuren, Belgium; MNHU – Museum für Naturkunde, Berlin, Germany; NM – KwaZulu-Natal Museum, Pietermaritzburg, South Africa; NZM – Livingstone Museum, Livingstone, Zambia; ROM – Royal Ontario Museum, Toronto, Canada; SMF – Senckenberg Museum, Frankfurt, Germany; SMNS – Staatliches Museum für Naturkunde, Stuttgart, Germany; TM – Ditsong National Natural History Museum (formerly Transvaal Museum), Pretoria, South Africa. Afrotropical taxa examined in the present study included: *O. madagascariensis* (Madagascar) and *O. martiensseni s.l.* (Africa, including animals that previous studies assigned to *O. icarus*) (Fig. 1, Table 1). Holotypes examined in the present study included: *O. icarus* (BMNH 16.10.9.1); *O. martiensseni* (MNHU 97523); and *O. madagascariensis* (type locality south of Soalala, Namoroka, Réserve naturelle intégrale no. 8,

Madagascar, MNHN 1953-1590). The crania of all type specimens examined were intact, with the exception of that of *O. icarus*, from which the mandible was missing (this was excluded from craniodental analyses).

Each specimen was assigned to a relative age class (1–6) based on cusp degradation of maxillary molars, and skull size and shape, before being measured (for ageing criteria, see the Supporting information, Appendix S2). To avoid the confounding effects of age on sample variation, only adult specimens assigned to age classes 4–5 were utilized in the study. Three morphometric data sets (craniodental measurements, dorsal, and ventral landmark data) were recorded for *Otomops* crania.

Morphological data of *Otomops w*ere pooled into three operational taxonomic units (OTUs), according to the phylogeographical patterns and genetic clades described by Lamb *et al*. (2008): (1) Ethiopia, Kenya, and Yemen, hereafter referred to as the north-eastern or NE OTU; (2) Burundi, Ivory Coast, South Africa, Tanzania, and Zimbabwe, herein termed the southeast-central-west or SECW OTU; and (3) Madagascar, hereafter referred to as the Malagasy OTU (Fig. 1, Table 1). Specimens from collecting localities that were not represented in the genetic-based study were assigned to an OTU based on their geographical origin and included: NE OTU – Djibouti; SECW OUT – DR of Congo, Malawi, Uganda, and Zambia. The holotypes of *O. martiensseni* and *O. icarus* were assigned to the SECW OTU.

Table 1. Geographic origin, OTU assignment and sample size for the specimens examined in this study.

			TM		Dors	Vent	PLS	
Species	Country	OTU	M	F	M	F	M	F
Otomops martiensseni s.l.	Burundi	S	1	0	1	0	1	0
-"-	Central African Republic	S	1	0	1	0	1	0
-"-	DR of Congo	S	1	1	1	1	1	1
-"-	Ivory Coast	S	2	1	2	1	2	1
-"-	Malawi	S	0	1	0	1	0	1
-"-	South Africa	S	18	22	26	23	16	19
-"-	Tanzania	S	2	0	2	0	2	0
-"-	Uganda	S	0	1	0	1	0	1
-"-	Zambia	S	1	0	1	0	1	0
-"-	Zimbabwe	S	1	0	1	0	1	0
	Total		27	26	35	27	25	23
Otomops martiensseni s.l.	Djibouti	N	0	1	0	1	0	1
-"-	Ethiopia	N	9	10	9	9	9	9
-"-	Kenya	N	24	28	25	26	25	21
-"-	Yemen	N	5	2	5	2	5	2
	Total		38	41	39	38	39	32
O. madagascariensis	Madagascar	M	18	20	19	19	-	-
Combined total			83	87	93	84	64	55

M = male, F = female, TM = traditional morphometrics, Dors = geometric morphometrics, dorsal view, Vent = geometric morphometrics, ventral view, PLS = partial least squares analysis and regression analysis. OTU: S = south-east-central-west OTU; N = north-eastern OTU; M = Malagasy OTU. Geographic localities are depicted in Fig. 1 and further details are provided in Appendix S1.

Traditional morphometrics

A total of 170 specimens (*N* = 83 males; *N* = 87 females) were examined (Table 1). Twelve craniodental measurements following Freeman (1981), were recorded from *Otomops* by LRR using Mitutoyo callipers accurate to 0.01 mm: GSL – greatest skull length; BCH – braincase height; BCB – braincase breadth; MB – mastoid breadth; ZB – zygomatic breadth; IOW – inter-orbital width; PL – palatal length; MTR – maxillary toothrow length; UCW – maxillary intercanine width; TBL – tympanic bulla length; LTR – mandibular toothrow length; MAT – moment arm of temporalis. A single external measurement, forearm length (FA), was measured from dried skins and fluid preserved study specimens examined by LRR.

Because Afrotropical *Otomops* display significant morphological sexual dimorphism (Fenton *et al*., 2002), we conducted a two-way multivariate analysis of variance (MANOVA) on craniodental variables to test for heterogeneity in sexual dimorphism amongst the three OTUs defined in the present study. This would determine whether morphological data of males and females could be combined in subsequent analyses.

ANOVA was used to test for significant size differences between the three Afrotropical OTUs. Descriptive statistics (mean, SD, and range) were computed for each OTU. Student–Newman–Keuls multiple range tests were used to identify statistically nonsignificant subsets of the three taxa. Canonical variates analysis (CVA) of $\log_{10}$-transformed variables was used to explore patterns of cranial variation among and between Afrotropical OTUs. Discriminant function analysis (DFA) using cross-validation was used to assess the validity of the *a priori* OTU assignment of specimens from the various geographical localities (Fig. 1, Table 1). All statistical analyses were conducted using IBM SPSS Statistics, version 19.0.0 (SPSS, Inc., 2010).

Geometric morphometrics

A total of 175 dorsal images (93 males; 82 females) and 163 ventral images (79 males; 84 females) were analysed (Table 1). Dorsal and ventral images of *Otomops* skulls were captured using a Fujifilm Finepix S8100 digital camera mounted on a tripod with the lens facing downward (18x optical zoom, 5 megapixel resolution, macro function). To standardize specimen placement and facilitate repeatability, each skull was placed on a stage (square Perspex dish with graph paper and plasticine) before image capture. Fourteen dorsal and 16 ventral landmarks (see the Supporting information, Appendix S3) were recorded from the cranial images using the software TPSDIG, version 2.16 (Rohlf, 2010a). Repeatability tests for specimen placement and/or image capture and landmark placement were conducted using the approach of Fadda, Faggiani & Corti (1997). Analyses of dorsal and ventral data sets showed image capture and landmark placement digitizing error levels were low compared to the resolution needed for diagnosing OTUs (data not shown).

TPSRELW, version 1.45 (Rohlf, 2010b) was used to conduct a generalized Procrustes analysis (GPA) of landmark data sets. Landmark configurations of each individual were translated, rotated, scaled, and superimposed to derive a consensus configuration of all specimens analysed. The GPA residuals variation was then decomposed into affine (UniX and UniY) and non-affine (partial warps) components of shape change. Shape matrices, consisting of both affine and non-affine shape components, were partitioned according to OTU. Partitioned shape matrices were analysed by means of CVA to explore patterns of intra and interspecific cranial shape variation. DFA using cross-validation was employed to test the validity of specimen taxonomic assignments. MANOVA (Wilks' lambda test criterion) tested the overall significance in shape variation between Afrotropical OTUs. All multivariate analyses were conducted in NYTSYS-PC, version 2.02k (Rohlf, 1999) or IBM SPSS Statistics. TPSREGR, version 1.37 (Rohlf, 2009) was used to obtain thin plate splines (deformation grids describing shape changes, magnified X 3) by regressing the original shape matrix onto the first and second projected canonical vectors. Centroid size (the square root of the sum of squares of the distances between each landmark and centroid), used as a geometrical estimate of cranial size, was extracted for each individual using TPSRELW. A statistical difference in $\log_{10}$-transformed centroid size of OTUs was tested with ANOVA. TPSREGR was used to regress the dorsal and ventral shape matrices against $\log_{10}$-transformed centroid size to test for allometry in shape data of males and females. A Goodalls' *F*-test was used to evaluate the statistical significance of the regression model and the explained variance was used to determine the overall fit of the model (Monteiro, 1999).

The relationship between cranial size of Afro-Arabian (sub-Saharan Africa, including the Arabian Peninsula) *Otomops* and ecogeographical variables was assessed using stepwise regression analysis. Ecogeographical variables (WORLDCLIM database, version 1.4; Hijmans *et al*., 2005) sampled at a spatial grid resolution of 30 arc seconds (approximately 1 km^2), were obtained for 28 localities for males ($N = 64$) and 24 localities for females ($N = 55$) (data set available from LRR) using the ArcView 3.2 extension BIOCLIMav (Moussalli, 2003). The *O. icarus* holotype was excluded from stepwise regression and partial least squares (PLS) analyses as the precise type locality was not fully specified. Ecogeographical variables included: Altitude; Bio1 (annual mean temperature); Bio4 (seasonality of temperature); Bio5 (maximum temperature of warmest month); Bio6 (minimum temperature of coldest month); Bio12 (annual precipitation); Bio13 (precipitation of wettest month); Bio14 (precipitation of driest month); and Bio15 (seasonality of precipitation). All variables were $\log_{10}$-transformed and screened for outliers and normality before conducting the statistical analysis. Principal components analysis (PCA) of the correlation matrix of ecogeographical variables was employed to derive a reduced set of factors that were independent of each other. Stepwise regression analysis was used to assess the influence of each ecogeographical factor on cranial size (as estimated by $\log_{10}$-transformed dorsal and ventral centroid size), whereas a two block PLS analysis (Rohlf, 2006) was utilized to explore the covariation between cranial shape and

the ecogeographical factors derived from PCA. The association between shape and ecogeographical datasets was measured by the amount of covariance explained by the first pair of PLS vectors (Rohlf & Corti, 2000). The significance of the correlations between PLS vector pairs was tested using permutation tests with 999 randomizations.

RESULTS

Geographic variation in sexual dimorphism

Two-way MANOVA on $\log_{10}$-transformed craniodental characters revealed a highly significant effect of OTU ($\lambda = 0.03$, $F_{24,306} = 65.54$, $P < 0.0001$), sex ($\lambda = 0.18$, $F_{12,153} = 59.89$, $P < 0.0001$), and OTU X sex interaction ($\lambda = 0.71$, $F_{24,306} = 2.38$, $P < 0.001$). Hence, we treated males and females separately in univariate and multivariate analyses. Analyses of the three morphological data sets for males and females produced congruent results. For practical reasons, only the results of analyses using craniodental measurements, dorsal landmark data of males, and ventral landmark data of females are presented. Results not presented are available from LRR.

Analyses based on the three Afrotropical OTUs

One-way ANOVA revealed that craniodental measurements, forearm length, male dorsal centroid size, and female ventral centroid size differed significantly between the three Afrotropical OTUs defined above (Tables 2, 3). Individuals of the NE OTU were significantly larger than the SECW animals, with animals from the Malagasy OTU being the smallest. Characters demonstrating the highest level of variation within Afrotropical males as indicated by *F*-values were BCH, ZB, FA, and GSL. Characters with high *F*-values in females were mostly width-related (MB, ZB, IOW) with the exception of GSL and LTR. The three OTUs were significantly different from each other for all variables, except UCW and MAT in males and UCW in females. MANOVA revealed significant OTU size differentiation for males ($\lambda = 0.03$, $F_{24,138} = 27.53$, $P < 0.0001$) and females ($\lambda = 0.01$, $F_{24,146} = 53.66$, $P < 0.0001$).

Table 2. Mean ± SD and range of external, craniodental and dorsal centroid size parameters of male Afrotropical *Otomops* classified to three Operational Taxonomic Units (OTUs) and results of ANOVA.

	Malagasy OTU		South-east-central-west OTU		North-eastern OTU		
Character	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	*F*–value
Greatest Skull Length (GSL)	25.7 ± 0.48 (18)	24.9–26.5	27.5 ± 0.54 (27)	26.5–28.8	28.5 ± 0.36 (38)	27.8–29.3	189.16
Braincase Height (BCH)	8.2 ± 0.20 (18)	7.9–8.5	8.6 ± 0.25 (27)	8.2–9.0	9.4 ± 0.18 (38)	8.9–9.8	243.84
Mastoid Breadth (MB)	12.6 ± 0.22 (18)	12.2–13.0	13.4 ± 0.34 (27)	12.1–13.8	13.9 ± 0.18 (38)	13.5–14.3	163.33
Zygomatic Breadth (ZB)	12.8 ± 0.26 (18)	12.3–13.2	14.0 ± 0.33 (27)	13.4–14.4	14.5 ± 0.23 (38)	14.0–14.9	227.14
Inter-orbital Width (IOW)	5.4 ± 0.22 (18)	4.9–5.8	6.2 ± 0.22 (27)	5.7–6.5	6.4 ± 0.19 (38)	6.0–6.8	159.99
Braincase Breadth (BCB)	10.5 ± 0.25 (18)	10.1–10.9	11.1 ± 0.38 (27)	10.6–12.1	11.6 ± 0.21 (38)	11.2–12.1	97.72
Anterior Palatal Length (PL)	10.3 ± 0.25 (18)	9.9–10.8	11.2 ± 0.48 (27)	10.4–12.0	11.5 ± 0.26 (38)	10.8–12.0	72.15
Maxillary Toothrow Length (MTR)	9.5 ± 0.25 (18)	9.1–10.0	10.2 ± 0.36 (27)	9.7–10.7	10.5 ± 0.21 (38)	10.1–11.0	84.08
Maxillary Inter-canine Length (UCW)	2.8 ± 0.24 (18)	2.4–3.2	**3.1 ± 0.27** (27)	**2.8–3.6**	**3.2 ± 0.18** (38)	**2.9–3.7**	21.08
Mandibular Toothrow Length (LTR)	10.1 ± 0.21 (18)	9.7–10.4	11.0 ± 0.32 (27)	10.6–11.6	11.4 ± 0.19 (38)	10.9–11.8	168.11
Moment Arm of Temporalis (MAT)	4.8 ± 0.18 (18)	4.4–5.1	**5.4 ± 0.26** (27)	**4.9–5.7**	**5.4 ± 0.16** (38)	**5.1–5.7**	57.27
Tympanic Bulla Length (TBL)	6.3 ± 0.26 (18)	5.9–6.7	6.7 ± 0.20 (27)	6.3–7.1	7.1 ± 0.17 (38)	6.7–7.5	94.44
Forearm Length (FA)	63.3 ± 1.12 (19)	61.0–66.0	66.0 ± 1.27 (25)	63.0–68.0	70.8 ± 1.69 (38)	68.4–75.6	192.97
Dorsal Centroid Size (DCZ) (log_{10}–transformed)	3.02 ± 0.01 (19)	3.00–3.03	3.04 ± 0.01 (35)	3.01–3.06	3.07 ± 0.01 (39)	3.06–3.08	145.61

All *F*–values were significant at the $P < 0.0001$ level. Statistically non-significant subsets ($P > 0.05$) based on Student Newman-Keuls tests are in bold and are underlined. Sample size of each OTU is provided in parentheses.

Table 3. Mean ± SD and range of external, craniodental and ventral centroid size parameters of female Afrotropical *Otomops* classified to three Operational Taxonomic Units (OTUs) and results of ANOVA.

	Malagasy OTU		South-east-central-west OTU		North-eastern OTU		
Character	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	*F*–value
Greatest Skull Length (GSL)	23.7 ± 0.48 (20)	22.8–24.5	25.5 ± 0.38 (26)	24.7–26.1	27.1 ± 0.41 (41)	26.2–27.9	438.74
Braincase Height (BCH)	7.9 ±0.17 (20)	7.6–8.2	8.3 ± 0.23 (26)	7.8–8.8	9.1 ± 0.16 (41)	8.7–9.5	281.68
Mastoid Breadth (MB)	11.9 ± 0.24 (20)	11.5–12.4	12.8 ± 0.20 (26)	12.3–13.1	13.5 ± 0.14 (41)	13.1–13.8	414.88
Zygomatic Breadth (ZB)	12.1 ± 0.24 (20)	11.6–12.6	13.2 ± 0.24 (26)	12.7–13.6	13.9 ± 0.22 (41)	13.4–14.3	359.89
Inter-orbital Width (IOW)	5.1 ± 0.15 (20)	4.9–6.4	5.9 ± 0.18 (26)	5.7–6.5	6.2 ± 0.14 (41)	5.9–6.6	344.36
Braincase Breadth (BCB)	10.1 ± 0.29 (20)	9.7–10.6	10.8 ± 0.22 (26)	10.2–11.1	11.4 ± 0.21 (41)	11.0–11.9	200.02
Anterior Palatal Length (PL)	9.3 ± 0.27 (20)	8.7–9.7	10.3 ± 0.28 (26)	9.8–10.8	10.7 ± 0.28 (41)	10.2–11.3	176.72
Maxillary Toothrow Length (MTR)	8.8 ± 0.21 (20)	8.4–9.1	9.5 ± 0.19 (26)	9.2–10.0	10.0 ± 0.22 (41)	9.5–1–10.5	246.18
Maxillary Inter-canine Length (UCW)	2.5 ± 0.14 (20)	2.3–2.8	**2.9 ± 0.18** (26)	**2.7–3.3**	**2.9 ± 0.18** (41)	**2.4–3.3**	36.06
Mandibular Toothrow Length (LTR)	9.2 ± 0.19 (20)	8.7–9.6	10.2 ± 0.24 (26)	9.7–10.7	10.7 ± 0.21 (41)	10.1–11.1	333.20
Moment Arm of Temporalis (MAT)	4.3 ± 0.12 (20)	4.0–4.5	4.9 ± 0.14 (26)	4.7–5.2	5.1 ± 0.13 (41)	4.9–5.4	271.20
Tympanic Bulla Length (TBL)	6.0 ± 0.24 (20)	5.6–6.5	6.4 ± 0.20 (26)	6.1–6.8	6.8 ± 0.19 (41)	6.4–7.1	96.46
Forearm Length (FA)	61.3 ± 1.22 (15)	60.0–63.5	63.3 ± 1.62 (19)	60.0–66.0	69.4 ± 1.69 (36)	65.7–72.7	175.02
Ventral Centroid Size (VCZ) (log_{10}–transformed)	2.98 ± 0.01 (19)	2.97–3.00	3.01 ± 0.01 (27)	2.99–3.03	3.05 ± 0.01 (38)	3.03–3.06	383.84

All *F*–values were significant at the $P < 0.0001$ level. Statistically non-significant subsets ($P > 0.05$) based on Student Newman-Keuls tests are in bold and are underlined. Sample size of each OTU is provided in parentheses.

CVA of craniodental measurements provided strong support for the morphological differentiation of the three Afrotropical OTUs (Fig. 2). Canonical variate 1 (CV1) was considered an indicator of overall size, with most variables displaying high and positive loadings in both male and female datasets. The three OTUs were clearly separated along CV1, which accounted for 88.3% and 85.0% of the morphological variation in males and females, respectively (Fig. 2, Table 4). The SECW OTU separated from the NE OTU along CV1 and CV2 in both the male and female plots. Based on factor loadings for CV2 (Table 4), SECW specimens possessed shorter braincases and larger MAT relative to NE animals. In addition, NE males had narrower inter-orbitals and proportionately shorter mandibular toothrows than SECW males. Cross-validated classification showed 100% correct taxonomic assignment of males and 98.9% correct assignment of females.

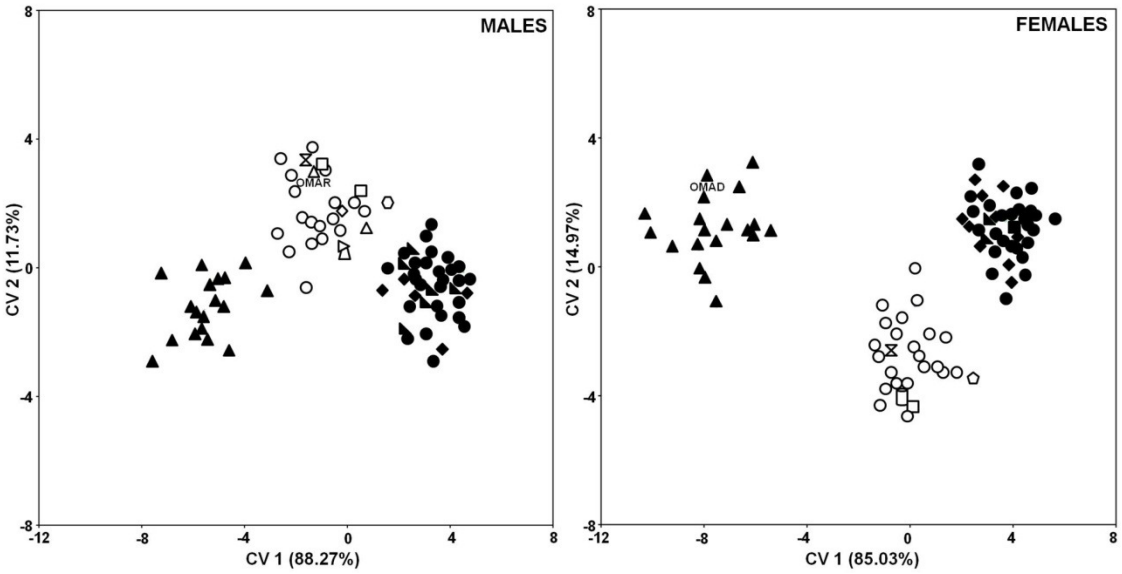

Figure 2. The first two canonical variates (CV) from a canonical variates analysis of $\log_{10}$– transformed craniodental variables of the three Afrotropical OTUs for males and females. Sample sizes for male and female datasets provided in parentheses. Malagasy OTU (18,20): ▲, Madagascar. South-east-central-west OTU (27,26): ▶, Burundi; ⬯, Central African Republic; ⧗, DR of Congo; ◻, Ivory Coast; ◘, Malawi; ○, South Africa; ▲, Tanzania; ⬠, Uganda; ◆, Zambia; ⏢, Zimbabwe. North-eastern OTU (38,41): ■, Djibouti; ◆, Ethiopia; ●, Kenya; ◣, Yemen. Types: OMAD = *O. madagascariensis*; OMAR = *O. martiensseni*.

Table 4. Variable-canonical vector correlation coefficients for the first two canonical variates from canonical variates analyses of 12 $\log_{10}$–transformed craniodental measurements recorded from males and females of the three Afrotropical OTUs.

Character	Males (n = 83)		Females (n = 87)	
	CV1	CV2	CV1	CV2
Greatest Skull Length (GSL)	0.621	0.250	0.713	0.273
Braincase Height (BCH)	0.686	–0.363	0.536	0.481
Mastoid Breadth (MB)	0.569	0.074	0.692	0.198
Zygomatic Breadth (ZB)	0.674	0.382	0.691	0.053
Inter-orbital Width (IOW)	0.558	0.477	0.660	–0.048
Braincase Breadth (BCB)	0.451	0.027	0.481	0.160
Anterior Palatal Length (PL)	0.379	0.259	0.467	–0.009
Maxillary Toothrow Length (MTR)	0.413	0.177	0.548	0.110
Maxillary Inter-canine Length (UCW)	0.201	0.201	0.205	–0.156
Mandibular Toothrow Length (LTR)	0.576	0.432	0.642	–0.049
Moment Arm of Temporalis (MAT)	0.311	0.435	0.578	–0.203
Tympanic Bulla Length (TBL)	0.440	0.070	0.333	0.148
Eigenvalue	12.322	1.639	20.185	3.553
Variance Explained (%)	88.268	11.732	85.033	14.967

MANOVA of landmark data revealed significant differences in cranial shape between OTUs defined for male (dorsal shape: λ= 0.02, $F_{48,134}$ = 18.45, P < 0.0001) and female (ventral shape: λ= 0.02, $F_{56,108}$ = 12.07, P < 0.0001) Afrotropical *Otomops*. The CV plots based on landmark data showed clear separation between the Malagasy, SECW, and NE OTUs (Fig. 3). Thin plate splines associated with the negative *x*-axis of CV1 in the respective male and female biplots demonstrated that Malagasy *Otomops* crania were distinguished from mainland specimens by narrow yet prognathic rostra, small nasals, an inward displacement of the zygomaxillary junction, outwardly-angled zygoma, expanded braincases with broad and outwardly directed bullae, a distinctly pointed supraoccipital region, and a larger occipital foramen. Thin plate splines associated with the positive *x*-axis of CV1 in male and female biplots indicated that the NE OTU was characterized by short and broad rostra, large nasals, a narrow braincase, elongated bullae, an outward deflection of the zygomaxillary junction, and posterior displacement of ventral landmark 14 (margin of hard palate). The SECW OTU was distinguished from the NE OTU by a wider post-orbital constriction, smaller nasals, and smaller bullae, as determined by the splines associated with the positive *y*-axis of CV2 in the male biplots and the negative *y*-axis of CV2 in the female biplot. Cross-validated DFA revealed that 98.9% of males and 91.7% of females were assigned correctly. Misclassified specimens included: one SEWC male classified to the NE OTU; one SEWC female assigned with the NE OTU; three SEWC females classified to the Malagasy OTU. Both the *O. icarus* and *O. martiensseni* holotypes were assigned to the SECW OTU. Regression of shape data onto centroid size revealed a significant correlation in males (dorsal: Goodalls' $F_{24,1488}$ = 6.11, P < 0.0001) and females (ventral: Goodalls, $F_{28,1484}$ = 9.67, P < 0.0001). The influence of allometry on shape variation was, however, marginal, accounting for 14.6% and 13.7% of total sample

variation in dorsal male and ventral female shape datasets, respectively (Cardini & O'Higgins, 2004; Colangelo *et al.*, 2010).

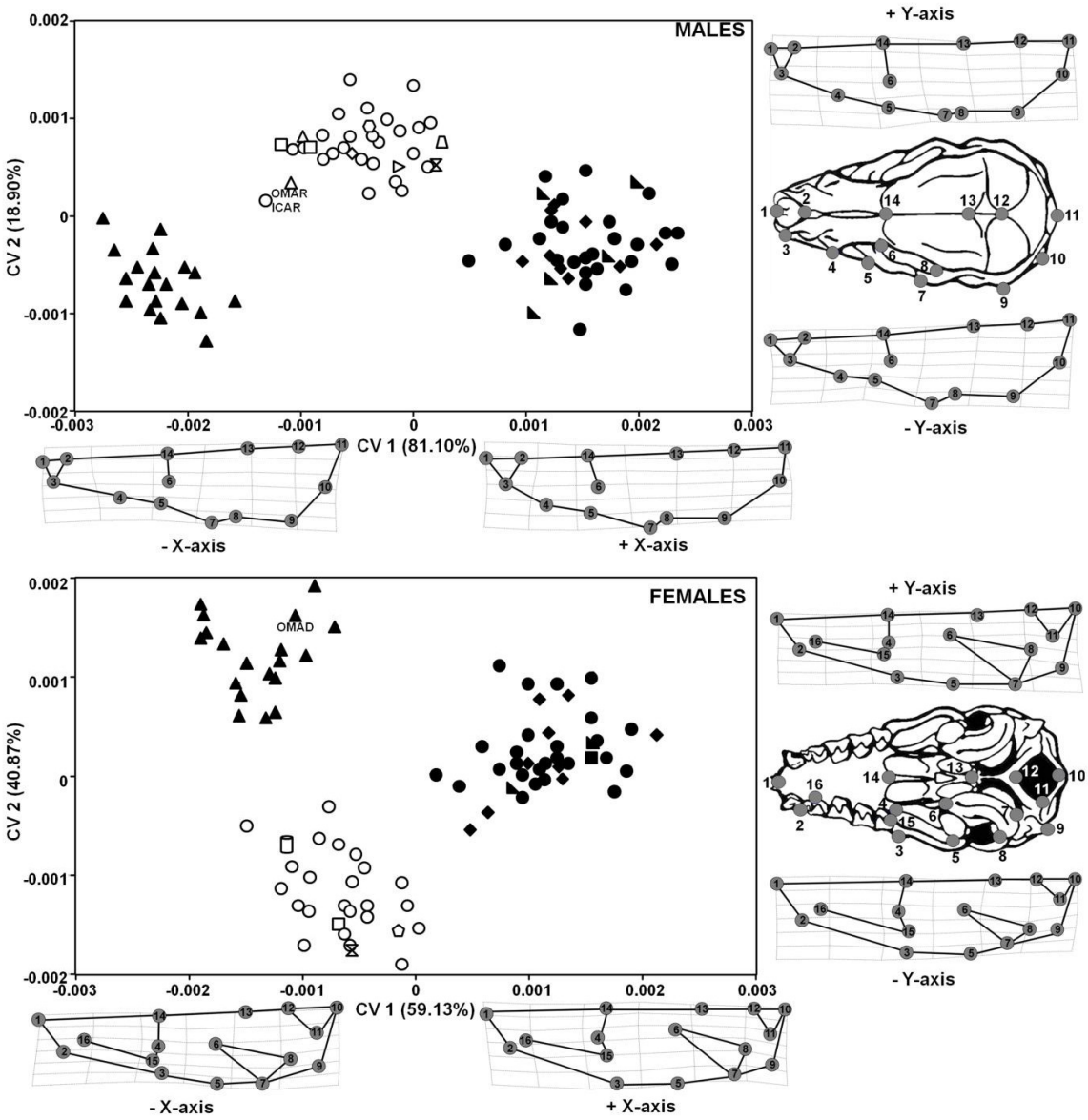

Figure 3. The first two canonical variates (CV) from a canonical variates analysis of landmark data of the three Afrotropical OTUs of males and females. Sample sizes for male and female datasets provided in parentheses. Malagasy OTU (19, 19): ▲, Madagascar. South-east-central-west OTU (35, 27): ▶, Burundi; ⬯, Central African Republic; ⧗, DR of Congo; □, Ivory Coast; ▭, Malawi; ○, South Africa; △, Tanzania; ⬠, Uganda; ◆, Zambia; ▱, Zimbabwe. North-eastern OTU (39, 38): ■, Djibouti; ◆, Ethiopia; ●, Kenya; ◣, Yemen. Types: OICA = *O. icarus*; OMAD = *O. madagascariensis*; OMAR = *O. martiensseni*.

Influence of ecogeographical factors on cranial size and shape

PCA reduced the nine ecogeographical variables to three factors with eigenvalues > 1 that combined, explained 91.3% and 86.1% of the variance in environmental variables among localities for males and females, respectively (Table 5). We interpreted the components as follows. The first principal component (PC1) was associated with seasonality and altitude because altitude, seasonality of precipitation, and precipitation in the driest month contributed to most of the variance observed along this axis. Annual mean temperature, maximum temperature of the warmest month, and minimum temperature of the coldest month contributed the most to the second component; thus, PC2 was associated with temperature. PC3 was linked with precipitation because precipitation of the wettest month and annual precipitation were highly correlated with this axis.

Table 5. Factor loadings of nine log_{10}–transformed ecogeographic variables on the first three principal components (PC) with eigenvalues >1 from 28 localities for male and 24 localities for female Afro-Arabian *Otomops*. Variable-factor correlations with magnitudes greater than 0.700 are indicated in bold.

Bioclimatic variables	Males			Females		
	PC1	PC2	PC3	PC1	PC2	PC3
Altitude	**0.942**	–0.207	0.052	**0.880**	0.031	–0.072
Mean Annual Temperature	–0.018	**0.959**	0.228	–0.110	**0.950**	–0.039
Maximum Temperature of warmest month	0.358	**0.878**	–0.104	0.322	**0.867**	–0.291
Minimum temperature of coldest month	–0.054	**0.748**	0.542	– 0.103	**0.807**	0.344
Seasonality in temperature	–0.590	–0.075	–0.695	–0.503	–0.590	–0.282
Annual Precipitation	–0.575	0.027	**0.786**	–0.514	0.083	**0.829**
Precipitation of wettest month	0.189	0.226	**0.925**	0.422	0.014	**0.892**
Precipitation of driest month	**–0.813**	–0.428	–0.035	**–0.873**	–0.143	0.037
Seasonality in precipitation	**0.956**	0.242	0.047	**0.960**	–0.057	0.112
Eigenvalue	3.989	2.683	1.546	3.526	2.503	1.722
Variance explained (%)	44.318	29.807	17.178	39.179	27.808	19.137

Stepwise regression analyses revealed the principal predictor of cranial size variation was PC1 in both male and female datasets (Table 6). Female cranial size was also shown to be negatively correlated with precipitation. The PLS analysis showed significant associations between cranial shape and ecogeographical variables. The first pair of PLS vectors explained 69.5% ($r = 0.75$, $P < 0.0001$) and 81.8% ($r = 0.84$, $P < 0.0001$) of total covariation between the two datasets for males and females, respectively. PC1 was strongly correlated with both the PLS shape vector (males: $r = 0.75$, $P < 0.0001$; females: $r = 0.75$, $P < 0.0001$) and PLS ecogeographical vector (males: $r = 0.99$, $P < 0.0001$; females: $r = 0.99$, $P < 0.0001$).

Table 6. Results of stepwise regression analyses of overall cranial size of male and female Afro-Arabian *Otomops* (as represented by $\log_{10}$–transformed dorsal and ventral centroid size) against ecogeographic variables.

Gender	Principal Component	β	*t*–value	*P*–value
Males (n = 64)	PC1 (altitude + seasonality)	0.748	8.882	< 0.0001
R^2 = 0.560	PC2 (temperature)	0.076	0.895	0.374 NS
adjusted R^2 = 0.553	PC3 (precipitation)	0.049	0.575	0.567 NS
Females (n = 55)	PC1 (altitude + seasonality)	0.783	9.902	< 0.0001
R^2 = 0.675	PC2 (temperature)	0.100	1.277	0.207 NS
adjusted R^2 = 0.663	PC3 (precipitation)	–0.250	–3.165	0.003

The standardized regression coefficients (β) reflect the explanatory power of individual predictor variables when other entered variables are held constant. The *t*–tests (*t*) provide details of the significance of predictor variables. R^2 – coefficient of multiple determinations when all predictor variables are entered. NS = non-significant.

The ordination of individuals along the PLS shape and ecogeographical vectors (Fig. 4) reflected similar groupings amongst male and female Afro-Arabian *Otomops*, as reported above. Thin plate splines depicting the mean shape of both OTUs demonstrated that individuals of the NE OTU have narrow post-orbitals (distance between dorsal landmarks 6 and 14), long frontals, a long hard palate, and elongated bullae. SEWC animals possessed longer rostra, broad post-orbitals, small nasals, outwardly-directed bullae, and shorter palates. Regression of shape matrices onto centroid size demonstrated that cranial shape variation amongst Afro-Arabian *Otomops* was significantly correlated with cranial size in males (dorsal: Goodalls' $F_{24,1488} = 6.11$, $P < 0.0001$) and females (ventral: Goodalls' $F_{28,1484} = 9.67$, $P < 0.0001$). Size-related shape variation, however, only accounted for 9.0% and 15.4% of total sample variation in dorsal male and ventral female shape datasets, respectively.

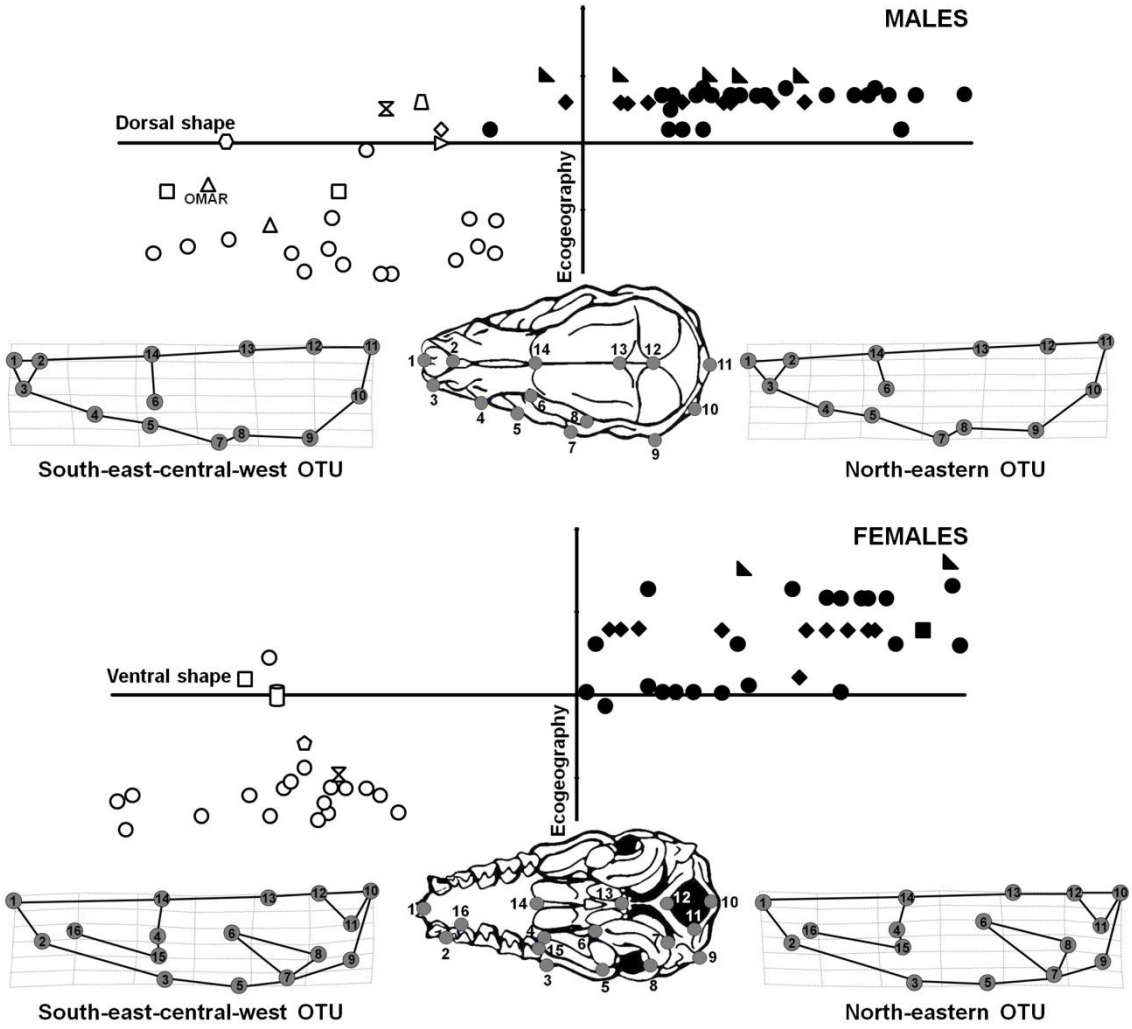

Figure 4. The first two latent vectors from a partial least squares analysis, demonstrating covariation patterns between cranial shape and ecogeographic factors in Afro-Arabian *Otomops*. Explained covariance = 69.7% (males), explained covariance = 81.8% (females). Sample sizes for male and female datasets are provided in parentheses. South-east-central-west OTU (28, 22): ▶, Burundi; ⬡, Central African Republic; ⧗, DR of Congo; □, Ivory Coast; ▭, Malawi; ○, South Africa; △, Tanzania; ⬠, Uganda; ◇, Zambia; ⏢, Zimbabwe. North-eastern OTU (42, 33): ■, Djibouti; ◆, Ethiopia; ●, Kenya; ◣, Yemen. Type: OMAR = *O. martiensseni*

DISCUSSION

Taxonomy and distributional limits of Afrotropical taxa

Analyses of traditional morphometric data and dorsal and ventral landmark data were congruent in revealing morphological divisions amongst Afrotropical *Otomops*. Multivariate analyses of craniodental measurements and landmark data, including leave-one out cross-validations, provided strong support for three geographically distinct OTUs corresponding to the genetic lineages described by Lamb *et al*. (2008): Malagasy *Otomops*, which is referred to as *O. madagascariensis*; *Otomops* from southern, eastern, central, and western Africa; *Otomops* from north-east Africa and the Arabian Peninsula. The findings of the present study corroborate the views of Peterson *et al*. (1995), who treated *Otomops* from DR of Congo, South Africa, Uganda, and Zimbabwe as a separate taxon from Kenyan *Otomops*. Traditional and geometric morphometric data indicates the range of the SECW OTU to extend from Ivory Coast in the west, to South Africa in the south, with its eastern most limits along the Albertine Rift of Burundi and Uganda, and the Eastern Arc Mountains of Tanzania. It incorporates the type localities of *O. martiensseni* and *O. icarus*. Hence, we consider *icarus* as a junior synonym of *martiensseni* and describe the range of the SECW OTU as that of *O. martiensseni s.s*. Additional material from western and central sub-Saharan Africa is required to clarify the distributional limits of *O. martiensseni s.s*. *Otomops* from Djibouti, Ethiopia, Kenya, and Yemen constitute a morphologically and genetically cohesive group that does not appear to have an available name and requires a formal taxonomic description.

Functional cranial morphology

Malagasy *Otomops* were significantly smaller than mainland animals in overall body size, exhibiting a 4% reduction in forearm length relative to SEWC individuals and a 11% reduction in forearm length relative to the NE OTU. Crania of Malagasy bats were distinctly shorter and narrower than their Afro-Arabian congeners, averaging 7% and 10% smaller in cranial size (as estimated by GSL) than SEWC and NE specimens, respectively. Similar patterns of morphological differentiation between closely-related mainland and insular bat populations have been described (Russo *et al*., 2009). For example, the morphological divergence of the Hawaiian hoary bat, *Lasiurus cinereus semotus*, from the mainland subspecies *L. c. cinereus*, was accompanied by an overall reduction in body and cranial size (Jacobs, 1996). Structural changes of the cranium and mandible associated with mastication have allowed the Hawaiian bat to prey upon smaller and/or hard-bodied insects not consumed by *L. c. cinereus*, a lepidopteran specialist (Jacobs, 1996; Barclay, Fullard & Jacobs, 1999). Similarly, *O. madagascariensis* has a varied diet, consuming almost equal volumes of hard-bodied

(Coleoptera) and soft-bodied (Lepidoptera and smaller-sized Diptera) prey (Andriafidison *et al*., 2007). This is in contrast to the two larger mainland taxa which feed predominantly on moths (Rydell & Yalden, 1997; M. C. Schoeman, unpubl. data).

Our geometric morphometric analyses demonstrated significant cranial shape divergence between insular and mainland Afrotropical bats and provided possible insights into interspecific differences in functional cranial morphology. Crania of *O. madagascariensis* were characterized by narrow, prognathic rostra and smaller nasals. This species possesses outwardly-angled zygoma, possibly allowing for greater masseter muscle attachment and hence increased crushing power during mastication (Maynard-Smith & Savage, 1959). In addition, the proportionately broader braincase coupled with a more posteriorly positioned supraoccipital margin in Malagasy *Otomops* compared to Afro-Arabian animals, suggests a greater surface area for the insertion of the medial and deep temporalis muscles (Freeman, 1981; Reduker, 1983; Nogueira, Peracchi & Monteiro, 2009). The temporalis is largely responsible for movement of the mandible during mastication and in resisting stress forces produced by captured hard-bodied insects (Maynard-Smith & Savage, 1959). Similar relationships between cranial morphology, size, and insertion of the masseter and temporalis muscles, including prey selection, have been described for *Myotis* spp. (Reduker, 1983), and phyllostomid bats (Nogueira *et al*., 2009). The broadening of the braincase in Malagasy *Otomops* also appears to be coupled to the posterior inflation and lateral shift of the tympanic bullae; morphological changes corresponding to the external auditory meatus. It has been suggested that changes in bulla shape and volume amongst taxa may correspond to adaptive differences in sound frequency sensitivity and recognition (Schleich & Vassalo, 2003; Colangelo *et al*., 2010). Hence, differences in bulla configuration between *O. madagascariensis* and mainland African animals may equip Malagasy *Otomops* to detect a wider variety of prey species than the Afro-Arabian taxa.

Ecophenotypic significance of cranial size and shape variation within Afro-Arabian *Otomops*

Forearm length and craniodental data revealed that north-eastern individuals were significantly larger in size than SECW OTU. This size disparity, however, was unable to explain most of the cranial shape variation between the NE and SEWC OTUs, suggesting that other factors such as ecogeographical variables may influence morphological variation in Afro-Arabian *Otomops*. Cranial size and shape variation in Afro-Arabian *Otomops* were significantly correlated with altitude, seasonality of precipitation, and precipitation of the driest month. Because these three ecogeographical variables are strongly correlated, it is difficult to determine the independent effects of each variable. In general, larger animals, particularly those belonging to the NE OTU, inhabit semi-arid, high altitudinal areas (> 900 m a.s.l.) characterized by low levels of precipitation in the dry months and pronounced seasonality in rainfall.

Increases in mammalian body and cranial size are often attributed to an ecophenotypic adaptive response to increase fasting endurance; this may be advantageous at high altitudes or semi-arid environments where primary productivity (as measured by annual precipitation) varies seasonally, rendering resources scarce (Lindstedt & Boyce, 1985; Millar & Hickling, 1990). Fasting endurance has been the proposed adaptive mechanism explaining intraspecific variation in body and/or cranial size in various mammalian species (Ritke & Kennedy, 1988; Gür, 2010). Similarly, increased body size in the tropical bat species *Cynopterus sphinx*, was associated with increasing seasonality of precipitation, and including decreased relative humidity and increased daily minimum temperature (Storz *et al*., 2001). Bats have low metabolic water reserves relative to their evaporative surface areas, most notably those of the wings (Thomas & Cloutier, 1992). Increases in chiropteran body size, correlated with a lowered surface area to volume ratio, may reduce evaporative water loss in more arid, resource-limited environments (Maharadatunkamsi *et al*., 2003). Maintenance and conservation of metabolic reserves may be essential in the reproductive strategies of pregnant or lactating northeastern African *Otomops* females, particularly in habitats where resource availability is unpredictable and limited because of low levels of precipitation.

Phylogenetic history may also have contributed to the observed morphometric patterns within Afro-Arabian *Otomops*. The congruence between the results obtained in the present study and those reported previously Lamb *et al*. (2008, 2011) suggests that the various structural components of *Otomops* crania may have, to some extent, retained a phylogenetic signal. Recovering similar patterns of structure in genetic and morphological datasets of closely related mammalian taxa is not an uncommon phenomenon (Cardini & O'Higgins, 2004; Cardini & Elton, 2008). This is particularly evident amongst recently divergent sister lineages, exhibiting 1–10% mitochondrial (mt) DNA sequence divergence (Caumul & Polly, 2005). Molecular dating, based on cytochrome *b* sequence data, places the divergence of Afro-Arabian *Otomops* clades approximately 1.2 Mya (0.7–1.8 Mya; 3.4% mtDNA divergence), coinciding with the Pleistocene (Lamb *et al*., 2008). Climatic oscillations and continued uplift of the East African Rift System during the late Pliocene and early Pleistocene initiated a progressive increase in aridity and seasonality of the eastern and north-eastern African palaeoenvironment (deMenocal, 2004; Sepulchre *et al*., 2006). Periods of intense aridification occurring approximately 2.8, 1.7, and 1.0 Mya (deMenocal, 2004) resulted in the fragmentation and isolation of populations of once widespread tropical species and the subsequent evolution of larger, arid and/or seasonally-adapted mammal taxa in eastern and north-eastern Africa (Renaud, Benammi & Jaeger, 1999; Bobe, Behrensmeyer & Chapman, 2002). It is therefore possible that morphological divergence in *Otomops* may be attributed to vicariant events leading to the separation of the two Afro-Arabian OTUs. This initial segregation may have been reinforced by subsequent directional selection, leading to the larger-sized north-east African individuals that are adapted to prolonged fasting-endurance in more seasonal climates, as well as smaller-sized *Otomops* in the relatively more mesic or buffered environments of southern, central, and western Africa.

In conclusion, the present study provides the first detailed cranial comparisons of Afrotropical *Otomops* using both traditional and geometric morphometric approaches. Our analyses delineated three well supported morphological groups of Afrotropical *Otomops* that correspond to the genetic lineages described by Lamb *et al*. (2008) and revealed several species-specific morphological traits. Morphometric patterns appear to reflect the phylogeography and ecophenotypic adaptations of Afrotropical *Otomops*. The relative contribution of each factor to morphological evolution within *Otomops* remains to be fully understood and explored.

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APPENDICES

APPENDIX ONE

List of specimens used in the morphometric analyses. The country, locality and museum catalogue number of each specimen is provided. Museum abbreviations: BMNH = Natural History Museum, London, United Kingdom; DM =Durban Natural Science Museum; Durban, South Africa; FMNH = Field Museum of Natural History, Chicago, USA; MNHN = Muséum National d'Histoire Naturelle, Paris, France; MRAC = Musée Royale d'Afrique Centrale, Antwerp, Belgium; MNHU = Museum für Naturkunde, Berlin, Germany; NM = KwaZulu-Natal Museum, Pietermaritzburg, South Africa; NZM = Livingstone Museum, Livingstone, Zambia; ROM = Royal Ontario Museum, Toronto, Canada; SMNS = Staatliches Museum für Naturkunde, Stuttgart, Germany; TM = Transvaal Museum, Northern Flagship Institution, Pretoria, South Africa. M = male, F = female, TM = traditional morphometrics, Dors = geometric morphometrics, dorsal view, Vent = geometric morphometrics, ventral view. OTU: S = south-east-central-west OTU; N = north-eastern OTU; M = Malagasy OTU.

Species	Country	Locality	Coordinates	Museum No.	Sex	Age class	OTU	TM	Dors	Vent
Otomops martiensseni s.l.	Burundi	2.3 km N, 0.7 km W Teza, Kibira	3°200' S, 0°550' E	FMNH 137633	M	5	S	X	X	-
-"-	Central African Republic	Bamingui-Bangoran NP	7°550' N, 19°290' E	BM 81.238	M	5	S	X	X	X
-"-	Côte d'Ivoire	Comoé NP	8°715' S, 3°797' W	SMF 92048	M	4	S	X	X	X
-"-	Côte d'Ivoire	Comoé NP	8°715' S, 3°797' W	SMF 92049	M	5	S	X	X	-
-"-	Côte d'Ivoire	Comoé NP	8°715' S, 3°797' W	SMF 92050	F	4	S	X	X	X
-"-	DR of Congo	Lufuko Stream, Marungu	7°400' S, 29°460'E	NZM 3395	M	5	S	X	X	X
-"-	DR of Congo	Welle River, Poko	3°080' N, 25°580' E	BM 19.3.92	F	4	S	X	X	X
-"-	Djibouti	Mount Day	11°460' N, 42°390' E	BM 69.1256	F	4	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 44.31328	M	4	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 64.36220	M	4	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 40.31315	M	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 46.31370	M	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 47.31371	M	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 48.31372	M	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 60.36217	M	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	SMF 41832	M	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	SMF 41833	M	5	N	X	X	-
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 61.36218	F	4	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 41.31316	F	5	N	X	-	-
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 42.31317	F	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 43.31318	F	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 45.31369	F	5	N	X	X	X

Appendix S1. Continued

Species	Country	Locality	Coordinates	Museum No.	Sex	Age class	OTU	TM	Dors	Vent
Otomops martiensseni s.l.	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 49.33964	F	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 50.33965	F	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 56.36213	F	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 57.36214	F	5	N	X	X	X
-"-	Ethiopia	Sof Omar Cave, Bale District	6°540' N, 40°480' E	HZM 63.36220	F	5	N	X	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 48666	M	4	N	X	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 48655	M	5	N	X	-	-
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 48656	M	5	N	X	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 63808	M	5	N	X	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 48660	M	5	N	-	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 48661	M	5	N	X	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 48663	M	5	N	-	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 48664	M	5	N	X	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 48667	M	5	N	X	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 63779	F	4	N	X	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 63782	F	4	N	X	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 63772	F	5	N	X	X	X
-"-	Kenya	19 km W of Makindu	2°180' S, 37°400' E	ROM 48654	F	5	N	X	X	X
-"-	Kenya	Chyulu Hills	2°350' S, 37°500' E	MRAC 38548	M	4	N	X	-	-
-"-	Kenya	Chyulu Hills	2°350' S, 37°500' E	MRAC 38549	M	4	N	X	X	X
-"-	Kenya	Chyulu Hills	2°350' S, 37°500' E	MRAC 38546	F	5	N	X	X	X
-"-	Kenya	Chyulu Hills	2°350' S, 37°500' E	MRAC 38547	F	5	N	X	X	X
-"-	Kenya	Chyulu Hills	2°350' S, 37°500' E	SMNS 46077	F	5	N	X	-	-
-"-	Kenya	Chyulu Hills	2°350' S, 37°500' E	SMNS 46079	F	5	N	X	-	-
-"-	Kenya	Ithundu Caves, Kiboko	2°120' S, 37°430' E	ROM 65876	M	4	N	X	X	X
-"-	Kenya	Ithundu Caves, Kiboko	2°120' S, 37°430' E	ROM 65877	M	5	N	X	X	X
-"-	Kenya	Ithundu Caves, Kiboko	2°120' S, 37°430' E	ROM 65875	F	5	N	X	X	X
-"-	Kenya	Ithundu Caves, Kiboko	2°120' S, 37°430' E	ROM 65878	F	5	N	-	X	X
-"-	Kenya	Ithundu Caves, Kiboko	2°120' S, 37°430' E	ROM 65879	F	5	N	X	X	X
-"-	Kenya	Ithundu Caves, Makindu	2°200' S, 37°420' E	ROM 81198	M	4	N	X	X	X
-"-	Kenya	Ithundu Caves, Makindu	2°200' S, 37°420' E	ROM 81199	M	5	N	X	X	-
-"-	Kenya	Lake Baringo, Kampi Ya Moto	0°260' N, 35°580' E	ROM 68362	F	4	N	X	X	X
-"-	Kenya	Lake Baringo, Kampi Ya Moto	0°260' N, 35°580' E	ROM 68364	F	4	N	X	X	X
-"-	Kenya	Lake Baringo, Kampi Ya Moto	0°260' N, 35°580' E	ROM 68360	F	5	N	X	X	X
-"-	Kenya	Lake Baringo, Kampi Ya Moto	0°260' N, 35°580' E	ROM 68366	F	5	N	X	X	X
-"-	Kenya	Machakos District	1°310' S, 37°160' E	MRAC 35264	F	4	N	X	X	X
-"-	Kenya	Makindu Cave, Makindu	2°180' S, 37°500' E	ROM 78158	M	4	N	X	X	X

Appendix S1. Continued

Species	Country	Locality	Coordinates	Museum No.	Sex	Age class	OTU	TM	Dors	Vent
Otomops martiensseni s.l.	Kenya	Makindu Cave, Makindu	2°180' S, 37°500' E	ROM 78155	M	5	N	X	X	-
-''-	Kenya	Makindu Cave, Makindu	2°180' S, 37°500' E	ROM 78157	M	5	N	X	X	X
-''-	Kenya	Makindu Cave, Makindu	2°180' S, 37°500' E	ROM 78156	F	4	N	-	X	X
-''-	Kenya	Makindu River	-	ROM 65873	F	5	N	X	X	X
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 36517	M	4	N	X	X	X
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 36519	M	4	N	-	-	X
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 91249	M	4	N	X	X	X
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 78151	M	5	N	X	X	-
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 78152	M	5	N	X	X	X
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 91250	M	5	N	X	X	X
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 41928	F	4	N	X	X	X
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 41932	F	4	N	-	X	X
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 78147	F	4	N	X	X	X
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 41920	F	5	N	X	X	-
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 41924	F	5	N	X	X	X
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 41927	F	5	N	X	X	-
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 78148	F	5	N	X	X	X
-''-	Kenya	Mount Suswa	1°090' S, 36°210' E	ROM 78154	F	5	N	X	X	-
-''-	Kenya	Nairobi	1°170' S, 36°490' E	ROM 79677	F	5	N	X	X	X
-''-	Kenya	Near Makindu, 192 km E of Nairobi	2°180' S, 37°500' E	ROM 48657	M	4	N	X	X	-
-''-	Kenya	Near Makindu, 192 km E of Nairobi	2°180' S, 37°500' E	ROM 48659	M	4	N	X	X	X
-''-	Kenya	Near Makindu, 192 km E of Nairobi	2°180' S, 37°500' E	ROM 48662	M	4	N	X	X	X
-''-	Malawi	Mangoche Mountain	14°270' S, 35°290' E	NZM 3228	F	4	S	X	X	X
O. icarus (holotype)	South Africa	Central Durban	29°510' S, 31°010' E	BM 16.10.9.1	M	4	S	-	X	X
Otomops martiensseni s.l.	South Africa	Hime Road, Berea, Durban	29°480' S, 31°010' E	DM 4950	M	4	S	-	X	X
-''-	South Africa	Durban	-	DM 5392	M	4	S	X	-	-
-''-	South Africa	296 Marine Drive, Brighton Beach,	29°540' S, 31°010' E	DM 5427	M	4	S	X	X	X
-''-	South Africa	Hillary, Durban	-	DM 5935	M	4	S	-	X	-
-''-	South Africa	Durban	-	DM 5936	M	4	S	-	X	-
-''-	South Africa	106 Bailey Road, Red Hill, Durban	29°460' S, 31°010' E	DM 6888	M	4	S	X	X	X
-''-	South Africa	137 Glenardle Road, Brighton Beach,	29°560' S, 30°000' E	DM 6930	M	4	S	X	X	X
-''-	South Africa	Durban	-	DM 7909	M	4	S	X	X	X
-''-	South Africa	Kingsway, Durban	30°230' S, 30°530' E	DM 7914	M	4	S	X	X	X
-''-.	South Africa	20 Jan Smuts Avenue, Northdene, Durban	-	DM 3886	M	5	S	X	X	X
-''-	South Africa	Ocean View Farm, Park Rynie, Durban	30°200' S, 30°220' E	DM 8032	M	4	S	X	X	X

Appendix S1. Continued

Species	Country	Locality	Coordinates	Museum No.	Sex	Age class	OTU	TM	Dors	Vent
Otomops martiensseni s.l.	South Africa	5 Springfield Drive, Westville, Durban	29°500' S, 30°933' E	DM 8571	M	4	S	-	X	X
-''-	South Africa	Queen Elizabeth Park, Pietermaritzburg	29°340' S, 30.190' E	DM 10790	M	4	S	X	X	X
-''-	South Africa	Durban North, Durban	-	DM 11731	M	4	S	X	X	X
-''-	South Africa	Percy Osbourne Road, Morningside, Durban	29°490' S, 31°010' E	DM 11732	M	4	S	X	X	X
-''-	South Africa	Fenniscowles Road, Umbilo, Durban	29°530' S, 30°580' E	DM 5344	M	5	S	X	X	X
-''-	South Africa	20 Jan Smuts Avenue, Northdene, Durban	-	DM 3885	M	5	S	-	X	-
-''-	South Africa	560 Marine Drive, Bluff, Durban	29°550' S, 31°000' E	DM 5509	M	5	S	-	X	X
-''-	South Africa	560 Marine Drive, Bluff, Durban	29°550' S, 31°000' E	DM 5511	M	5	S	-	X	X
-''-	South Africa	560 Marine Drive, Bluff, Durban	29°550' S, 31°000' E	DM 5512	M	5	S	-	X	-
-''-	South Africa	Park Rynie, Durban	30°180' S, 30°440' E	DM 5605	M	5	S	X	X	X
-''-	South Africa	50 Winifred Drive, St. Winifred, Durban	30°540' S, 30°510' E	DM 6220	M	5	S	X	X	X
-''-	South Africa	Durban	-	DM 6904	M	5	S	X	X	X
-''-	South Africa	27 Hunters Way, 412 Waterside, Umgeni Heights, Durban	29°480' S, 31°120' E	DM 10294	M	5	S	X	X	X
-''-	South Africa	Wentworth, Durban	-	HZM 1.2145	M	5	S	X	X	X
-''-	South Africa	Durban, South Africa	-	NM 379	F	4	S	-	X	X
-''-	South Africa	Marshall Grove, Carrington Heights, Durban	29°530' S, 30°580' E	DM 3518	F	4	S	-	X	X
-''-	South Africa	Umhlanga, Westbrooke, Durban	-	DM 4490	F	4	S	-	-	X
-''-	South Africa	296 Marine Drive, Bluff, Durban	29°540' S, 31°010' E	DM 5426	F	4	S	-	X	X
-''-	South Africa	560 Marine Drive, Bluff, Durban	29°550' S, 31°000' E	DM 5518	F	4	S	X	-	X
-''-	South Africa	Durban	-	DM 5936	F	4	S	-	X	X
-''-	South Africa	106 Bailey Road, Red Hill, Durban	29°460' S, 31°010' E	DM 6887	F	4	S	X	X	X
-''-	South Africa	La Lucia, Durban	-	DM 6936	F	4	S	X	X	X
-''-	South Africa	La Lucia, Durban	-	DM 6937	F	4	S	X	-	X
-''-	South Africa	27 Hunters Way, 412 Waterside, Umgeni Heights, Durban	29°480' S, 31°120' E	DM 8419	F	4	S	X	X	X
-''-	South Africa	27 Hunters Way, 412 Waterside, Umgeni Heights, Durban	29°480' S, 31°120' E	DM 10295	F	4	S	X	X	X
-''-	South Africa	29 Glen Anil Street, Glen Anil, Durban	29°450' S, 31°020' E	DM 11434	F	4	S	X	X	X
-''-	South Africa	3 km of Modimolle (Nylstroom)	24°660' S, 28°130' E	DM 11526	F	4	S	X	X	X
-''-	South Africa	Voortukker Strand, near Margate, Durban	30°510' S, 30°220' E	HZM 4.3078	F	4	S	X	X	-
-''-	South Africa	Bluff, Durban	-	TM 38865	F	4	S	X	-	-
-''-	South Africa	26 Waller Crescent, Berea, Durban	29°490' S, 31°000' E	DM4760	F	5	S	X	X	X
-''-	South Africa	296 Marine Drive, Brighton Beach, Durban	29°540' S, 31°010' E	DM 5425	F	5	S	X	X	X
-''-	South Africa	560 Marine Drive, Bluff, Durban	29°550' S, 31°000' E	DM 5514	F	5	S	X	X	X
-''-	South Africa	560 Marine Drive, Bluff, Durban	29°550' S, 31°000' E	DM 5516	F	5	S	X	X	X
-''-	South Africa	106 Bailey Road, Red Hill, Durban	29°460' S, 31°010' E	DM 6886	F	5	S	X	X	X

Appendix S1. Continued

Species	Country	Locality	Coordinates	Museum No.	Sex	Age class	OTU	TM	Dors	Vent
Otomops martiensseni s.l.	South Africa	Ocean View Farm, Park Rynie, Durban	30°200' S, 30°220' E	DM 8031	F	5	S	X	X	X
-''-	South Africa	27 Hunters Way, 412 Waterside, Umgeni Heights, Durban	29°480' S, 31°120' E	DM 8420	F	5	S	X	X	X
-''-	South Africa	8 Buys Road, Pinetown, Durban	29°450' S, 30°370' E	DM 8421	F	5	S	X	-	X
-''-	South Africa	Voortukker Strand, near Margate, Durban	30°510' S, 30°220' E	HZM 3.3077	F	5	S	X	X	X
-''-	South Africa	Bluff, Durban	-	TM 33867	F	5	S	X	-	-
-''-	South Africa	Bluff, Durban	-	TM 42514	F	5	S	X	-	-
-''-	South Africa	Wentworth, Durban	-	HZM 1.2145	M	5	S	X	X	X
-''-	South Africa	Durban		NM 378	M	5	S	-	X	X
-''-	Republic of Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15°.466'N, 43°550' E	HZM 51.33976	M	4	N	X	X	X
-''-	Republic of Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15°466' N, 43°550' E	HZM 53.33978	M	5	N	X	X	X
-''-	Republic of Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15°466' N, 43°550' E	HZM 54.33979	M	5	N	X	X	X
-''-	Republic of Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15°466' N, 43°550' E	SMF 87648	M	5	N	X	X	X
-''-	Republic of Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15°466' N, 43°550' E	SMF 87649	M	5	N	X	X	-
-''-	Republic of Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15°466' N, 43°550' E	HZM 55.33980	F	4	N	X	X	X
-''-	Republic of Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15°466' N, 43°550' E	HZM 39.31195	F	5	N	X	X	X
Otomops martiensseni holotype	Tanzania	Magrotto Plantation, Magrotto Hill, near Tanga	5°070' S, 38°030' E	MNHU 97523	M	5	S	X	X	X
Otomops martiensseni s.l.	Tanzania	Tongwe F.R., Tanga, Muheza District	5°305' S, 38°728' E	SMF 79542	M	4	S	X	X	X
-''-	Uganda	Budongo Forest, Bunyoro	1°450' S, 31°350' E	ROM 46695	F	4	S	X	X	X
-''-	Zambia	Mafinga Mountains	10°250' S, 33°500' E	Unaccessioned specimen	M	5	S	X	X	X
-''-	Zimbabwe	Hostes Nicolle Institute, Sengwa Wildlife Ranch	18°167' S, 28°217' E	ROM 83979	M	5	S	X	X	X
Otomops madagascariensis	Madagascar	Province d'Antsiranana, RS d'Ankarana, 3.5 km SE Andrafiabe	12°942' S, 49°055' E	FMNH 176357	M	4	M	X	X	
-''-	Madagascar	Province d'Antsiranana, RS d'Ankarana, 3.5 km SE Andrafiabe	12°942' S, 49°055' E	FMNH 176356	M	4	M	-	X	
-''-	Madagascar	Province d'Antsiranana, RS d'Analamerana, Grotte de Barazibe	12°711' S, 49°473' E	FMNH 178849	F	4	M	X	X	X

Appendix S1. Continued

Species	Country	Locality	Coordinates	Museum No.	Sex	Age class	OTU	TM	Dors	Vent
Otomops madagascariensis	Madagascar	Province d'Antsiranana, RS d'Analamerana, Grotte de Barazibe	12°711' S, 49°473' E	FMNH 178850	F	4	M	X	X	X
-''-	Madagascar	Province d'Antsiranana, RS d'Analamerana, Grotte de Barazibe	12°711' S, 49°473' E	FMNH 178851	F	5	M	X	X	X
-''-	Madagascar	Province d'Antsiranana, RS d'Ankarana, Grotte Boribe	13°000' S, 49°000' E	FMNH 183896	F	4	M	X	X	X
-''-	Madagascar	Province d'Antsiranana, RS d'Ankarana, Grotte Boribe	13°000' S, 49°000' E	FMNH 183897	F	4	M	X	X	X
-''-	Madagascar	Province d'Antsiranana, RS d'Ankarana, Grotte Boribe	13°000' S, 49°000' E	FMNH 183927	F	5	M	X	X	X
-''-	Madagascar	Province d'Antsiranana, RS d'Ankarana, Grotte Antsiroandoha	12°891' S, 49°098' E	FMNH 177398	F	5	M	X	X	X
-''-	Madagascar	Province de Fianarantsoa, 3.8 km NW Ranohira, along Namaza River	22°540' S, 45°380' E	FMNH 166073	F	4	M	X	X	X
-''-	Madagascar	Province de Mahajanga, Grotte d'Anjohibe, 3.7 km NE Antanamarina	15°537' S, 46°886' E	FMNH 179318	F	4	M	X	X	X
-''-	Madagascar	Province de Mahajanga, Grotte d'Anjohibe, 3.7 km NE Antanamarina	15°537' S, 46°886' E	FMNH 179316	F	5	M	X	X	X
-''-	Madagascar	Province de Mahajanga, Grotte d'Anjohibe, 3.7 km NE Antanamarina	15°537' S, 46°886' E	FMNH 179317	F	5	M	X	X	X
-''-	Madagascar	Province de Mahajanga, Parc National de Bemahara, Grotte d'Anjohimbabazimba	18°245' S, 44°716' E	FMNH 169667	M	4	M	-	X	X
-''-	Madagascar	Province de Mahajanga, Parc National de Bemahara, Grotte d'Anjohimbabazimba	18°245' S, 44°716' E	FMNH 169692	M	5	M	X	X	X
-''-	Madagascar	Province de Mahajanga, Parc National de Bemahara, Grotte d'Anjohimbabazimba	18°245' S, 44°716' E	FMNH 169693	F	5	M	X	X	X
-''-	Madagascar	Province de Mahajanga, Parc National de Bemahara, Grotte d'Anjohimbabazimba	18°245' S, 44°716' E	FMNH 169689	F	5	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23°540' S, 43°767' E	FMNH 172397	M	4	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23°540' S, 43°767' E	FMNH 172938	M	4	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23°540' S, 43°767' E	FMNH 172942	M	4	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23°540' S, 43°767' E	FMNH 172934	M	5	M	X	X	X

Appendix S1. Continued

Species	Country	Locality	Coordinates	Museum No.	Sex	Age class	OTU	TM	Dors	Vent
Otomops madagascariensis	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23°540' S, 43°767' E	FMNH 172936	M	5	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23°540' S, 43°767' E	FMNH 172939	M	5	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23°540' S, 43°767' E	FMNH 172940	M	5	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23°540' S, 43°767' E	FMNH 172941	M	5	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St. Augustin	23°548' S, 43°716' E	FMNH 172947	M	5	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St. Augustin	23°548' S, 43°716' E	FMNH 172948	M	4	M	X	X	X
	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St. Augustin	23°548' S, 43°716' E	FMNH 172951	M	4	M	X	-	-
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St. Augustin	23°548' S, 43°716' E	FMNH 172950	M	5	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St. Augustin	23°548' S, 43°716' E	FMNH 172945	F	4	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St. Augustin	23°548' S, 43°716' E	FMNH 172949	F	4	M	X	X	X
	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St. Augustin	23°548' S, 43°716' E	FMNH 172949	F	4	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St. Augustin	23°548' S, 43°716' E	FMNH 172952	F	4	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St. Augustin	23°548' S, 43°716' E	FMNH 172943	F	5	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St. Augustin	23°548' S, 43°716' E	FMNH 172944	F	5	M	X	X	X
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St. Augustin	23°548' S, 43°716' E	FMNH 172953	F	5	M	X	X	X
Otomops madagascariensis holotype	Madagascar	Province de Mahajanga, Réserve Naturelle (intégrale no. 8) au Sud du Soalala, Namoroka	16°230' S, 45°280' E	MNHN.CG 1953-1	F	5	M	X	X	X

APPENDIX TWO

Specimen ageing criteria

The first maxillary molar (M^1) located on the right-hand side of *Otomops* skulls was primarily used in describing the degree of cusp degradation per specimen. This is the first of three maxillary molars to erupt and thus acquires the greatest degree of wear of all three molars. The degree of development and degradation on the second (M^2) and third (M^3) molars were also recorded and used in the age-categorization of specimens. Specimens belonging to relative age classes 1 to 3 were not considered in this study as they represented juveniles. As specimens belonging to age class 6 were poorly represented in both male and female data sets, they were incorporated into the age class 5; thus analyses were primarily conducted on adult specimens belonging to toothwear classes 4 and 5.

Class 1: M^1 and M^2 partially erupted; M^3 absent or partially erupted, no cusp wear, incisors and premolars curved inwards; distinct sutures, braincase round with no supraoccipital ridge; sagittal crest absent or underdeveloped; wide interorbital width relative to greatest skull length (present in toothwear class 1 and 2). **Class 2:** M^1 and M^2 fully erupted; M^3 present yet partially erupted; minimal to no wear of cusps; incisors curved slightly inwards; distinct sutures; braincase round with no supraoccipital ridge; sagittal crest absent or underdeveloped; wide interorbital width relative to greatest skull length (present in toothwear class 1 and 2). **Class 3:** all molars fully erupted; cusp 1 of M^1 interlocked with cusp 3 of M^2; slight wear on M^1 and M^2 cusps; braincase edge rounded; slight development of supraoccipital ridge and sagittal crest; wide interorbital width relative to greatest skull length; interorbital ridges developing. **Class 4:** all molars fully interlocked; M^1 and PM^2 interlocked; moderate wear on all molars; dentine exposed on occlusional surfaces of molars; braincase edge ovoid; supraoccipital ridge and sagittal crest well developed; interorbital ridges present; narrow interorbital constriction relative to greatest skull length and braincase breadth (present in toothwear class 4-5). **Class 5:** heavy wear on cusps of M^1 and M^2; moderate to heavy wear on cusps of M^3; dentine exposed on occlusional surfaces of molars; braincase edge ovoid; supraoccipital ridge and sagittal crest well-developed; interorbital ridges present and well-defined; narrow interorbital constriction relative to greatest skull length and braincase breadth (present in toothwear class 4-6). **Class 6:** extremely heavy wear on cusps of M^1 and M^2; heavy wear on cusps of M^3; dentine exposed on occlusional surfaces of molars; braincase edge ovoid; supraoccipital ridge and sagittal crest well developed; interorbital ridges present and well-defined; narrow interorbital constriction relative to greatest skull length and braincase breadth (present in toothwear class 4-6).

APPENDIX THREE

Description of landmarks recorded on the dorsal and ventral views of *Otomops* crania (as depicted in Fig. 1). Numbers in parentheses provide type of landmark. Type 1 - juxtaposition of cranial regions; Type 2 - extremities of bony or dental processes; Type 3 – extremal points and maximum point of curvature (e.g. the bottom of a concavity).

Dorsal view. Landmark 1: anterior most point of nasals (2). Landmark 2: upper anterior point of nasals (2). Landmark 3: Anterior most point of premaxilla (2). Landmark 4: exterior tip of lachrymal process (2). Landmark 5: junction of jugal region of zygomatic arch and maxilla (1). Landmark 6: anterior point of interior orbit (2). Landmark 7: junction of jugal and squamosal process (1). Landmark 8: posterior point of interior orbit (2). Landmark 9: point of maximum curvature of mastoids (3). Landmark 10: edge of supraoccipital margin (2). Landmark 11: posterior-most point of supraoccipital (2). Landmark 12: junction of interparietal, parietal and sagittal sutures (1). Landmark 13: junction of frontal, parietal and sagittal sutures (1). Landmark 14: junction of frontal, nasal and sagittal sutures (1).

Ventral view. Landmark 1: anterior most point of nasals (2). Landmark 2: posterior border of maxillary canine alveolus (2). Landmark 3: junction of jugal region of zygomatic arch and maxilla (1). Landmark 4: anterior point of interior orbit (2). Landmark 5: posterior point of interior orbit (2). Landmark 6: anterior point of external bulla (2). Landmark 7: exterior point of external auditory meatus (2). Landmark 8: junction of internal and external bullae (1). Landmark 9: posterior point of occipital condyle (2). Landmark 10: posterior point of maximum curvature of occipital foramen (3). Landmark 11: Lateral edge of occipital foramen (2). Landmark 12: anterior most point of occipital foramen (3). Landmark 13: basisphenoid-basioccipital junction (1). Landmark 14: junction of posterior palate and midline (1). Landmark 15: posterior edge of M_3 (2). Landmark 16: anterior edge of M_1 (2)

CHAPTER FIVE

SUMMARY AND CONCLUDING COMMENTS

BEYOND DNA SEQUENCING

A dramatic increase in the application of DNA sequence data and the associated sophistication of phylogenetic techniques has addressed many long-standing evolutionary and ecological questions concerning the world's Chiroptera. In most cases, DNA sequence based phylogenies have detailed historical evolutionary processes that have a) provided a better understanding of contemporary patterns of diversity, b) directed efforts in the discovery of lineage-specific morphological attributes, and c) have been insightful for the interpretation of ancestral, independent and convergent character states in the evolution of taxa. Despite these advancements, there remains a general paucity of comprehensive and/or resolved phylogenies for a substantial portion of most taxonomic groups within the Afrotropics. This study employed comparative chromosome painting and geometric morphometric approaches as independent means to provide further insights into the systematics of Afrotropical bats. These approaches were specifically chosen for this investigation as they have been relatively under-utilised in evolutionary studies of regional bats, yet elsewhere in the world have provided valuable insights into cladogenic events formerly inferred from DNA sequence data (e.g. Evin *et al*. 2008, 2011; Sotero-Caio *et al*. 2010; Volleth *et al*. 2011).

CYTOTAXONOMY AND CHROMOSOMAL EVOLUTION WITHIN AFROTROPICAL CHIROPTERA

Basic karyotypic data are only available for half of the approximate 1260 described chiropteran species (Volleth 2013). Deficiencies in detailed karyotypic descriptions of Afrotropical bats has hampered our efforts in understanding chromosomal changes that may be coupled with important events in the evolutionary history of many taxa. This study provided G- and/or C-banded karyotypes for eight chiropteran species assigned to seven families, including the enigmatic and endemic *Myzopoda aurita* from Madagascar, for which high resolution chromosomal data was not available and/or uncertainty characterised their phylogenetic antiquity. Comparative chromosome painting experiments based on *Myotis myotis* paints revealed that Robertsonian (Rb) fusions and fissions are by far the most dominant structural rearrangement responsible for karyotypic differences amongst the taxa under study. This is not surprising as these are the most frequent rearrangements involved in genomic restructuring in bats (Bickham & Baker 1980) and mammals in general (Wienberg 2004; Ferguson-Smith & Trifinov 2007). A consequence of karyotypic evolution mediated via Rb rearrangements is the high incidence of convergent events due to identical arm combinations in distantly related taxa, which can limit the utility of chromosomal characters in resolving interfamily relationships amongst Chiroptera (Moa *et al*. 2007, 2008). This study also identified several convergent chromosomal characters amongst evolutionary distant taxa (Chapters 2 & 3).

Despite the prevalence of convergent cytogenetic characters and monobrachial homologies, a single chromosomal synapomorphy (MMY 9+11) was recovered that supported the assignment of *Myzopoda aurita* within the Noctilionoidea as suggested by DNA-based sequence analyses (Teeling *et al*. 2005; Miller-Butterworth *et al*. 2007). Comparative G-banding studies suggest this character is a common feature amongst phyllostomid bats (Baker & Bass 1979; Sotero-Caio e*t al*. 2011). Increased taxon sampling, in particular the monotypic Mystacinidae that is the proposed sister taxon to Myzopodidae and included within the Noctilionoidea, may be able to confirm whether MMY 9+11 does represent a synapomorphy of the superfamily and whether *Myzopoda* is in fact aligned with this predominantly Neotropical clade. Alternately, the synteny MMY 3+4 shared between the Vespertilionidae and *Myzopoda* may provide support for molecular hypotheses that place *M. aurita* within the Vespertilionoidea superfamily (Van den Bussche *et al*. 2003; Eick *et al*. 2005). Hence, the taxonomic affinities of Myzopodidae remain somewhat unclear and further studies are required before any definitive conclusion can be drawn on the phylogenetic placement of Myzopodidae within the bat family tree.

Despite the limitations of molecular cytogenetic techniques to fully resolve the phylogenomic placement of *Myzopoda*, it provided important insights into the karyotypic evolution of this distinctive bat family. One interesting aspect of karyotypic evolution within the Myzopodidae is the occurrence of a novel X-A translocation. Such rearrangements are considered rare amongst bats and have only been reported from the Phyllostomidae and Vespertilionidae (Volleth 1987; Volleth & Heller 2007). Although more refined investigations based on human-derived chromosomal probes are necessary to confirm the autosome translocated to the X-chromosome, it is clear that Myzopodidae represents the third known bat family to carry this rearrangement implicated in karyotype-mediated speciation (see White 1978). Sex-autosome translocations are not typically subject to convergence (Rokas & Holland 2000), and the formation of such unique rearrangements may be favoured by the presence of interstitial heterochromatic blocks (IHBs) that segregate the translocated segments (Parish *et al*. 2002).

Another point of interest is the possible occurrence of tandem fusions in the genomic evolution of *Myzopoda*. Very low diploid numbers, such as 2n = 26 in *M. aurita*, can only be explained by the involvement of non-Rb rearrangements such as tandem fusions (Bickham 1987; Pieczarka *et al*. 2005; Mao *et al*. 2008; Volleth & Eick 2012). Primitive chiropteran species, such as *Myzopoda*, may display a higher distribution of telomeric repeat sequences $(TTAGGG)_n$ or ITs within the telomeric and/or centromeric and interstitial chromosomal regions that may provide potential evolutionary breakpoints (Meyne 1990; Faria *et al*. 2009). Comparative genomic studies of other eutherian groups have shown that such evolutionary breakpoints regions are localised to specific chromosomal hotspots characterised by a high number of tandem repeats that are distributed heterogeneously throughout the genome and are commonly associated with fragile sites (Ruiz-Herrera *et al*. 2005, 2006; Ruiz-Herrera &

Robinson 2007). These break sites may allow for the establishment of Rb and tandem fusion products and/or inversions and, hence, can make for useful cytogenetic markers in subsequent phylogenomic studies where their presence can be confirmed through the use of chromosome-specific DNA repeat probes. Additional cytogenetic studies of *Myzopoda* will also aid in determining whether or not the evolutionary breakpoint in the MMY8 homologue, a cytogenetic feature previously considered confined to the Pteropodiformes and suggested to represent a synapomorphy uniting the Rhinolophoidea (Volleth *et al*. 2002; Ao *et al*. 2007; Mao *et al*. 2008), is a homoplastic character carried by *Myzopoda* and rhinolophoid bats. Recent preliminary painting studies have, however, shown that two MMY8 homologous subchromosomal elements are also present within the genomes of *Emballonura* and *Nycteris* (Volleth 2013). The chromosomal breakpoints of the homologues in these primitive Vespertilioniformes taxa differ from those found in the rhinolophoid bats. The presence of an X-A translocation and the possible occurrences of tandem fusions in the karyotypic evolution of *M. aurita* provide added evidence for the consideration of this deep-branching family as unique among Chiroptera. Of further interest would be a detailed karyotypic assessment of the sister species, *M. schliemanni*, to determine whether it exhibits similar chromosomal characteristics.

Chromosomal data confirmed various plesiomorphic characters described for the Pteropodiformes (Chapter 3). These data also demonstrated a close alliance between the pteropodine and rousettine fruit bats with their divergence described by approximately five major karyotypic differences that include Rb fusions/fissions, heterochromatic polymorphisms, and inversions. This study further highlights the relative importance of inversions in chromosomal evolution of pteropodids. The cryptic pericentric inversion detected on the MMY 4+19 homologue of *Pteropus rufus*, corresponding to HSA 3+21 homologous sequences (Volleth *et al*. 2002, 2011) and representing one of the largest proposed ancestral elements located within the chiropteran genome, requires further attention. Until the present study, only the closely-related rousettine species *Eonycteris spelaea* and *Rousettus leschenaulti* exhibited entire HSA 3+21 synteny conservation. Volleth *et al*. (2011) considered the arrangement in the rousettine bats as a derived state. A full cytogenetic survey of other pteropodid species from different genera is required to determine whether the MMY 4+19 syntenic arrangement in *P. rufus* constitutes a plesiomorphic state, and whether the proposed derived state is an synapomorphy of the rousettine clade as defined by molecular DNA studies (e.g. Giannini & Simmons 2005; Almeida *et al*. 2011).

With the application of MMY paints, this study revealed that the MMY16/17+24 synteny found in rousettine and hipposiderid bats (Chapter 3), sometimes considered a homoplastic feature present within both families (see Volleth *et al*. 2002; Ao *et al*. 2007), may in fact have different break / fusion points as indicated by painting results of *R. madagascariensis*. Comparisons between Afrotropical fruit bats and the Indomalaysian *Cynopterus* were limited due to the low number of shared syntenies between taxa. Painting analyses of additional species that display intermediate steps of chromosomal evolution between *Cynopterus* and

other pteropodids are needed to fully resolve the phylogenomic relationships amongst fruit bats. The inclusion of the Afrotropical *Eidolon dupreanum* and *E. helvum* in further chromosome painting studies may prove vital in understanding the complexities that underpin chromosomal evolution at the intergeneric level in this diverse chiropteran group.

Hipposideridae represents another taxonomic group wherein phylogenomic relationships amongst various genera and species have not been fully studied, as very few species have been examined using chromosome painting techniques. Painting results for one of the most basal taxa within the genus *Hipposideros*, *H. commersoni*, provided key insights into the proposed ancestral complement of the family. The syntenic associations of MMY 8+14 and MMY 7+19, proposed synapomorphies of Hipposideridae, were not present in the genome of *H. commersoni* yet are present in the genomes of other members of the genus and in *Aselliscus stoliczkanus* (AST). *Aselliscus stoliczkanus* has been proposed as possessing the most primitive hipposiderid chromosomal complement relative to other *Hipposideros* spp. as it shared three ancestral elements with pteropodids (MMY 10, MMY 12, MMY 23+13), including the identical G-banding pattern in the *p* arm of AST 11 homologous to MMY23. Until the present study, all *Hipposideros* spp. surveyed using chromosome painting techniques have displayed various paracentric inversion/s and heterochromatic addition in the short arm of the chromosome homologous to AST 11 that have led to altered G-banding patterns (Volleth *et al*. 2002; Mao *et al*. 2010). *Hipposideros commersoni* is the first known member of the genus that exhibits a G-banding pattern that is identical to both fruit bats and *A. stoliczkanus*. This brings into question whether the karyotype of *A. stoliczkanus* is truly representative of the ancestral hipposiderid karyotype.

The most recent molecular study of Afro-Arabian hipposiderid bats, provide strong support for the assignment of *A. stoliczkanus* at the terminal branch of a clade that is the successive sister lineage to *Hipposideros* (Benda & Vallo 2009). The study also suggested that *Hipposideros* arose before *Aselliscus*, although dating estimates may not be entirely accurate (Benda & Vallo 2009). Further efforts should be aimed at increasing the taxon sampling of Afrotropical hipposiderids in chromosome painting studies, particularly the inclusion of the genera *Cloeotis*, *Triaenops*, and *Paratriaenops* as these constitute a distinct tribe, Triaenopini (*sensu* Benda & Vallo 2009), that is well differentiated from the genera *Hipposideros*, *Asellia*, *Coelops*, and *Aselliscus*.

Overall, this study highlighted the limited use of chromosomal characters in phylogenomic investigations directed at the intrageneric level, with the notable exception of certain genera such as *Hipposideros* (Mao *et al*. 2010; this study) and *Rhinolophus* (Moa *et al*. 2008), due to the highly constrained nature of chromosomal evolution amongst Chiroptera despite deep genetic divergence amongst congenerics. Few interchromosomal rearrangements appeared to have occurred during the karyotypic evolution of the Malagasy Chiroptera studied herein and their extralimital congenerics. This is not surprising given the fact that karyotypic conservatism has been reported from a number of bat lineages (e.g. Baker & Patton 1967;

Bickham 1979a, b; Baker & Bickham 1980; Bickham *et al*. 1986; Sreepada *et al*. 2008). Thus, the present study does not provide support for the theory of speciation via chromosomal evolution, at least at the specific level. Hence, alternate taxonomic methods, including morphometric techniques, may prove useful to elucidate possible mechanisms responsible for species divergence at lower taxonomic levels.

ADAPTIVE CRANIAL EVOLUTION IN AFROTROPICALCHIROPTERA

The analyses of cranial morphology using traditional and geometric morphometric techniques provides the first detailed descriptions of cranial size and shape differences between *Otomops* spp. across their Afrotropical distributions (Chapter 4). Morphometric data were consistent in delineating three morphologically distinct species, previously described from genetic studies (Lamb *et al*. 2006, 2008, 2011). These data revealed that cranial divergence amongst Afro-Arabian taxa was strongly influenced by bioclimatic factors including altitude, seasonality of precipitation, and precipitation in the driest month. Based on morphometric patterns and molecular divergence estimates, it was established that morphological evolution within Afro-Arabian *Otomops* was correlated with the fluctuating palaeoclimate and the increasing aridity and seasonality over north-eastern Africa. The timing of speciation within the *Otomops* group approximately 1.2 Mya coincides with diversification events of other taxa across Africa subregion (see Bobe *et al*. 2002; deMenocal 2004). Previous studies have shown that both speciation and extinction are greatly influenced by dramatic changes in climate (Flagstad *et al*. 2001; Bobe & Behrensmeyer 2004; Maslin & Christensen 2007; Tolley *et al*. 2008).

In general, this study has contributed towards resolving the taxonomic status of Afrotropical *Otomops* and has provided a better understanding of the cranial variation between the taxa studied. It has also provided support for molecular studies and has identified morphological characters that may be used in taxonomic diagnoses. This investigation has also highlighted the importance of museum material and the maintenance of biological repositories as a vital component in the advancement of systematic studies of Afrotropical Chiroptera as demonstrated by recognition of a new and yet undescribed taxon from northeast Africa and the Arabian Peninsula. The extent of the range of the northeastern OTU and its conservation status requires further investigation. Preliminary studies have revealed subtle differences in the structure of the baculum of the two Afro-Arabian species (L.R. Richards, unpublished data). Other taxonomic markers, including karyotypic data, may provide additional support for the recognition of this distinct taxon.

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