INSIGHTS FROM AVIAN DIVERSIFICATION PATTERNS IN THE GUINEO-CONGOLIAN TROPICAL LOWLAND FORESTS

A Dissertation

by

JERRY WALLACE HUNTLEY

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Chair of Committee,	Gary Voelker
Committee Members,	Jessica Light
	Kevin Conway
	Gregory Sword
Head of Department,	Michael Masser

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ABSTRACT

The biogeographical history of the Afro-tropical Guineo-Congolian lowland forests during the Plio-Pleistocene is characterized by pervasive fragmentation-coalescence cycling due to global climatic oscillations. Vicariance scenarios driven by forest fragmentation have long been hypothesized as major mechanisms for the creation and maintenance of Afro-tropical avian diversity. However, the timing and center of diversification events remains unclear. Additionally, the current paradigm within the field regards the Guineo-Congolian forests as regions of little importance in creating genetic diversity patterns. The goal of this dissertation is to address, using multiple levels of evidence, potential avian diversification patterns across Sub-Saharan lowland tropical forests. Utilizing molecular data from 75 avian species, we undertook a combination of molecular and biogeographic methods to construct time-calibrated phylogenies, ancestral area estimations, haplotype networks, and diversification rate estimations.

We found substantial, geographically discrete genetic structuring in the majority of sampled avian species, much of it dating to the Pleistocene epoch. Additionally, ancestral area estimations reconstruct the lowland forests as the area of origin the ancestor of our two highest sampled genera. Diversification rates estimated for three genera recovered increasing diversification rates throughout the Plio-Pleistocene. Our results strongly indicate that lowland tropical forests are potentially harboring much more cryptic avian genetic diversity than previously hypothesized. The prevailing view

of the Guineo-Congolian forests as regions that have been responsible for the creation of genetic diversity does not appear to be appropriate for many of the avian species sampled here. Specifically, understory taxa, which are heavily tied to the dark, dense parts of the lowland tropical forests showed the most substantial levels of genetic distance between geographic regions. These results highlight the importance of considering behavioral ecology when studying the biogeographic history of forest taxa. Overall, the data from this study signals that climate-induced forest fragmentation in the Plio-Pleistocene undoubtedly played a substantial role in the cyclical creation of new avian diversity in the Guineo-Congolian forests of Africa.

DEDICATION

This dissertation is dedicated to my family: Scott, Terrie, Laurie, Kevin, Kristin, and Harper Huntley.

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Contributors

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NOMENCLATURE

ACO1	Aconitase 1 intron-10
ATP6	ATPase subunit 6
BAMM	Bayesian analysis of macroevolutionary mixtures
BEAST	Bayesian analysis of macroevolutionary mixtures
Bp	Base pairs
C	Congo forest block
CAR	Central African Republic
COI	Cytochrome oxidase I
CYTB	Cytochrome oxidase beta
DEC	Dispersal-extinction cladogenesis
DEC+J	Dispersal-extinction cladogenesis plus jump dispersal
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of the Congo
EG	Equatorial Guinea
EGG	Equatorial Guinea/Gabon
GTR	General time reversible
Ka	Thousand years ago
LG	Lower Guinean forest block
LRT	Likelihood ratio test
Ma	Million years ago

MCMC	Markov chain Monte Carlo
MSH	Montane Speciation Hypothesis
mtDNA	Mitochondrial deoxyribonucleic acid
MYO	Myoglobin intron 2
N	Number
ND2	NADH-dehydrogenase subunit 2
nuDNA	nuclear deoxyribonucleic acid
p	Pairwise
PCR	Polymerase chain reaction
PFRH	Pleistocene Forest Refuge Hypothesis
PP	Posterior probability
RBH	Riverine Barrier Hypothesis
RSA	Republic of South Africa
SD	Standard deviation
SLL	Sierra Leone/Liberia
TGF β2	Transforming growth factor beta 2
UG	Upper Guinean forest block

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CHAPTER I

INTRODUCTION

Early biogeographers revealed highly complex distributional patterns of avifauna within African lowland tropical forests, leading to substantial interest in discordant patterns throughout the region. (Diamond and Hamilton 1980; Crowe and Crowe 1982). These investigators relied on scenarios of vicariance and allopatric speciation created by the climatically-driven cyclical fragmentation of tropical forests during the Pleistocene epoch (1.8 Ma – 11,800 Ka). The primary hypothesis framing this scenario was the Pleistocene Forest Refuge Hypothesis (PFRH) (Haffer 1969, 1974) which was proposed specifically for the Afro-tropics by Mayr and O'Hara (1986).

However, the PFRH was based primarily on differences in plumage variation, leading to the exclusion of many widespread avian taxa lacking significant phenotypic differences. Therefore, an alternative refugial hypothesis, the Montane Speciation Hypothesis (MSH), shifted the center of diversification way from lowland forests and into montane tropical forests through the use of molecular data (Fjeldså 1994; Fjeldså & Lovett 1997; Roy 1997; Roy, Sponer & Fjeldså 2001; Fjeldså et al. 2007; Fjeldså & Bowie 2008). These studies found that the majority of avian montance taxa were relatively young (~ 5 Ma or younger) when compared with lowland forest taxa (~12-20 Ma), leading the authors to categorize the Afro-tropical lowland forests as regions where incapable of creating substantial recent genetic diversification.

These two hypotheses represent a polar view of tropical forests that have been historically common when biogeographers have attempted to explain discordant biodiversity patterns in tropical regions across the globe. Biogeographers have often attempted to simplify complex diversity patterns in the tropics by classifying them as either "evolutionary museums" or "evolutionary cradles" (Stenseth 1984; Jablonski 1993; Gaston and Blackburn 1996; Chown and Gaston 2000; Wiens and Donoghue 2004; Jablonski *et al.* 2006; McKenna and Farrell 2006; Moreau *et al.* 2013). Essentially, these biogeographers either viewed tropical regions as "cradles" which have given rise to new lineages *in situ*, or "museums" which have driven little genetic or morphological diversification.

The primary goal of this dissertation is to investigate potential avian diversification patterns within the Afro-tropics inside the framework of evaluating the role ("cradle" versus "museum") lowland forests have played in creating avian diversity. We approach this question by evaluating Afro-tropical avian biogeography on three levels. First, we evaluate a genus of birds containing species which inhabit both tropical forests and arid habitats in Sub-Saharan Africa, which may allow us to understand how historic forest change has affected species on the broadest (continental) level. Second, we study potential historic diversification patterns in two well sampled genera of understory birds for a narrower view of the effects of forest fragmentation. Last, we evaluate a large comparative genetic dataset of 75 avian forest species encompassing multiple levels of behavioral ecology. By examining potential patterns of diversity on these three levels, we may be able to shed light on the drivers of diversification within

lowland Afro-tropical forests and the ultimate role these regions have played throughout the climatic history of the continent.

CHAPTER II

A TALE OF THE NEARLY TAIL-LESS: THE SYSTEMATICS AND BIOGEOGRAPHY OF THE AVIAN GENUS *SYLVIETTA* (CROMBECS)[*]

II.1 Introduction

The past two decades have seen significant growth in our knowledge of the effect of global climatic oscillations on the habitats of the Sub-Saharan African continent during the Pliocene and Pleistocene epochs. Between 5 – 3.5 Ma (Pliocene) the global climate experienced a sustained increase in humidity, leading to substantial forest expansion in Equatorial Africa (Hamilton and Taylor 1991; Cane and Molnar 2001; Feakins *et al*. 2005; Sepulchre *et al*. 2006). Data from wind-blown dust and carbon isotopes demonstrate that the African climate became less humid starting in the mid to late Pliocene (~3.4-2.8 Ma; deMenocal 1995; Zachos *et al*. 2001; deMenocal 2004; Ravelo *et al*. 2004). During this climate shift there were perturbations between humid and dry conditions, with step-like shifts in the amplitude of overall aridity with peaks at 2.8 (±0.2), 1.7 (±0.1), and 1.0 (±0.2) Ma (de Menocal 2004). Impacts on the major Sub-Saharan biomes of Africa (i.e. tropical forests, and arid woodlands or savannah) were exemplified by cyclical bouts of retraction/fragmentation and subsequent expansions throughout the Plio-Pleistocene. The consequences of these habitat perturbations have been examined in regards to several mammalian, avian and reptilian taxa and yet our

[*] Reprinted with permission from "A tale of the nearly tail-less: The systematics and biogeography of the avian genus *Sylvietta* (Crombecs)" by Jerry Huntley and Gary Voelker, 2017. *Zoologica Scripta*, DOI: 10.1111/zsc.12240, Copyright 2017 by John Wiley and Sons.

knowledge of diversification and how vertebrate lineages achieved current distributions in the Afro-tropics is lacking, as compared to that of temperate regions (Hewitt 2004). In particular, the impacts of historic habitat fragmentation on avian diversity within Afro-tropical forests remains especially understudied.

However, we know that the wet-dry cycling of the African climate during the Plio-Pleistocene had drastic impacts on African tropical forests. Afro-tropical lowland and montane forests cycled between periods of retraction and fragmentation (peaks of aridity) in which forests survived in isolated pockets (refugia), followed by subsequent periods of expansion and coalescence (peaks of humidity) (Prigogine 1988; Maley 1996; Anhuf *et al*. 2006). Forest fragmentation/expansion cycling has long been surmised as a driving factor of Afro-tropical avian diversification, as forest taxa could have survived forest retraction events in isolated pockets of stable refugia, potentially leading to allopatric divergences (Haffer 1974; Mayr and O'Hara 1986; Roy 1997; Fjeldså and Lovett 1997; Roy *et al*. 2001; Fjeldså *et al*. 2005; Fjeldså *et al*. 2007; Fjeldså and Bowie 2008; Voelker *et al*. 2010).

Indeed, recent studies of Afro-tropical birds have discovered deep genetic divergences and discrete geographic structuring within several lineages (e.g., *Hylia prasina* (Marks 2010), and *Bleda syndactylus*, *Bleda canicapillus*, and *Bleda eximius* (Huntley and Voelker 2016)), reinforcing the potential significance of Plio-Pleistocene forest refuges as mechanisms for creating and maintaining diversity. Further reinforcing the importance of historic refugial forests as diversification centers, several mammalian taxa display similar patterns of genetic diversity in western and central Africa, to those

seen in birds (Nicolas *et al*. 2008; Bryja *et al*. 2010; Nicolas *et al*. 2011; Jacquet *et al*. 2015).

In contrast, as African tropical forests expanded during warmer and more humid interglacial periods, arid habitats (e.g. open woodlands, savannah, scrublands, etc.) retracted and survived in pockets of arid refugia, subsequently expanding as aridity increased and forests retracted (deMenocal 2004). Several studies on arid-adapted mammals have provided evidence for the size and location, as well as the potential importance of arid refugia in western, eastern, southern, and south-western Africa on creating genetic diversity in mammals (lions: Barnett *et al*. 2006; baboons: Zinner *et al*. 2009; review of several ungulate studies: Lorenzen *et al*. 2012). In addition, expansion of tropical forests to the east coast of Africa approximately 5-3.5 Ma (Hamilton & Taylor 1991; Cane & Molnar 2001; Feakins *et al*. 2005; Sepulchre *et al*. 2006) has been demonstrated as a primary driver of divergences between isolated northern and southern lineages in a growing number of arid-adapted avian groups (*Anthus*: Voelker 1999; *Motacilla*: Voelker 2002; *Monticola*: Outlaw *et al*. 2007; *Saxicola*: Illera *et al*. 2008; *Lanius collaris*: Fuchs *et al*. 2011; *Myrmecocichla*: Voelker *et al*. 2012; *Erythropygia* and allies: Voelker *et al*. 2014; *Muscicapa* and allies: Voelker *et al*. 2015). Furthermore, recent investigations of arid-adapted southern African taxa have demonstrated within-region inter and intra-specific divergences. For example, several studies have found substantial genetic diversity between populations within South Africa, despite the absence of any geographical barrier to gene flow (Outlaw *et al*. 2007; Ribeiro *et al*. 2011; Barlow *et al*. 2013). The patterns of divergence recovered within these taxa were

surmised to be related to various historical shifts in regional habitats resulting from cycling patterns of Plio-Pleistocene African aridity.

However, the overwhelming majority of investigations of the effects of climatic perturbations on the biogeographic history of African taxa have concentrated solely on either tropical or arid-adapted taxa. Few studies have examined widespread Sub-Saharan groups with members inhabiting both tropical forest and arid habitats. One widespread Sub-Saharan group of avian species whose collective ranges incorporate both lowland tropical and arid regions of Sub-Saharan Africa is the genus *Sylvietta* (the Crombecs). *Sylvietta* is composed of nine species inhabiting a complex variety of habitats throughout Sub-Saharan Africa (Fig. 1).

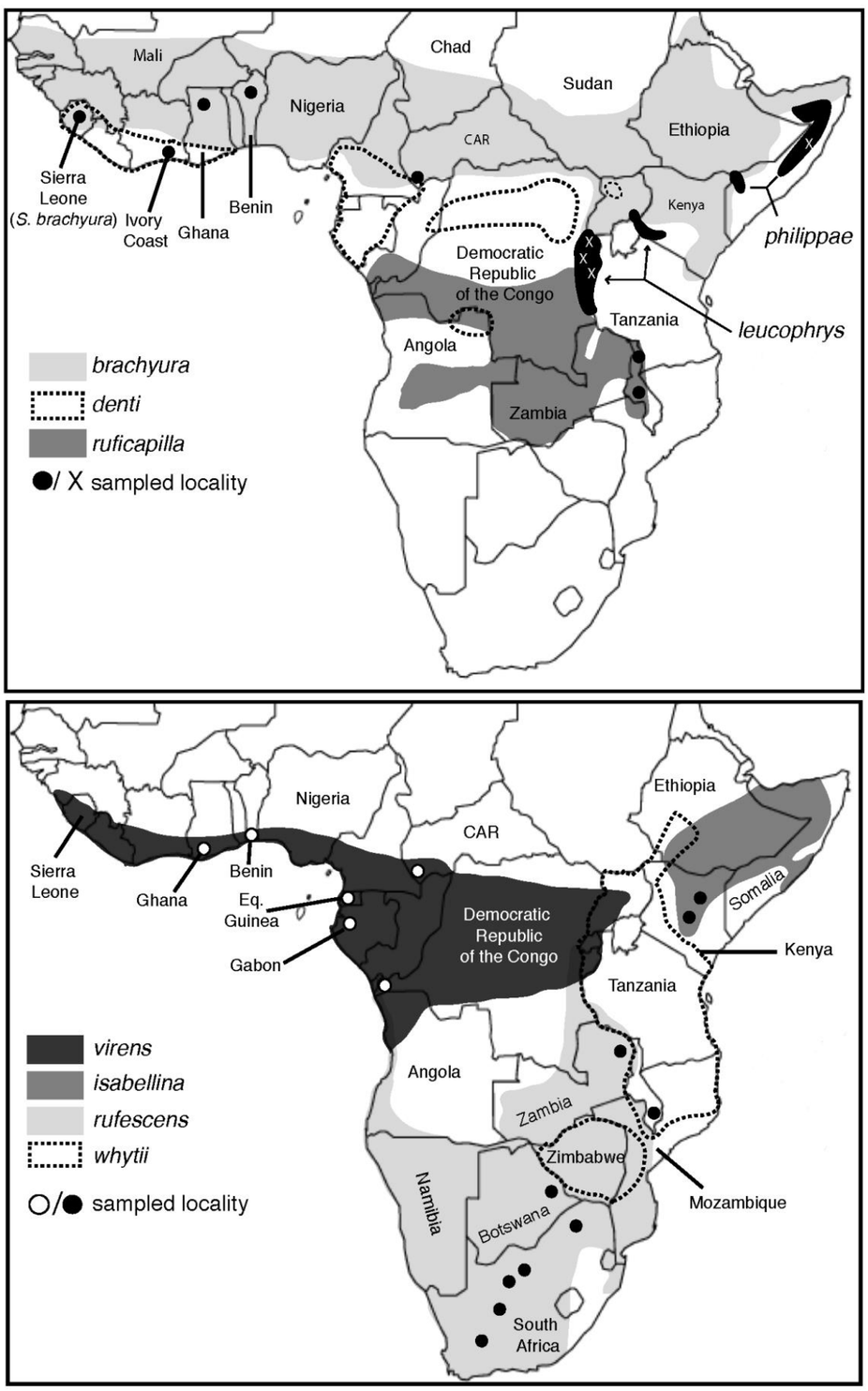


Figure 1. Range maps for currently recognized species within *Sylvietta* as well as general sampling points.

Two members of this genus, the Green Crombec (*Sylvietta virens*) and the Lemon-bellied Crombec (*Sylvietta denti*), inhabit the primary and secondary lowland forests of the Upper Guinean, Lower Guinean, and Congo Forest Blocks while another, the White-browed Crombec (*Sylvietta leucophrys*), inhabits montane forests in the Albertine Rift and northern sections of the Eastern Arc Mountains (Fig. 1). The remaining six species are arid/semi-arid adapted lineages which can be divided into three groups: (1) the Northern Crombec (*Sylvietta brachyura*) which is found in the dry Sahelian wooded savannahs north of the Afro-tropical forests, (2) Phillipa's Crombec (*Sylvietta philippae*) and the Somali Crombec (*Sylvietta isabellina*) which inhabit acacia/thorn scrub and semi-arid bush-land of Ethiopia and Somalia, and (3) a group of three taxa found in southern and eastern Africa (Fig. 1). Within the latter group, the Long-billed Crombec (*Sylvietta rufescens*) prefers drier savannah, mixed woodland, and open *Mopane* woodland in southern Africa (Fig. 1). The Red-capped Crombec (*Sylvietta ruficapilla*) inhabits wooded savannah and the edges of secondary forests directly to the south of the Congo Forest (Fig. 1). Finally, the Red-faced Crombec (*Sylvietta whytii*) prefers a variety of arid/semi-arid habitats with populations in the northern part of their range preferring woodland and thorn-scrub/acacia, while members in the southern part of the range prefer *Uapaca* riverine forests and *Brachystegia* (Fig. 1). Overall, the complex diversity and distributions of the genus *Sylvietta* make it an excellent model for further investigations of the importance of Plio-Pleistocene habitat cycling in shaping diversification patterns in Sub-Saharan avian lineages.

Further, no phylogenetic investigation exists examining the relationships among *Sylvietta* species, and early hypotheses of species relationships within the genus revolved around morphology, range and habitat data (Bairlein 2006). It has been posited that two superspecies groups exist within *Sylvietta*: one composed of *S. brachyura* + *S. whytii* + *S. philippae*, and one comprised of *S. rufescens* and *S. isabellina*. No strict hypothesis exists regarding the relationships of the remaining four species, though Irwin (1968) considered *S. ruficapilla* and *S. whytii* to be sibling species based on morphology and voice.

The goals of this study are three-fold. First, we undertake the first investigation of phylogenetic relationships within *Sylvietta*, using molecular markers (two mtDNA and two nuclear loci). Second, we use information yielded from molecular markers in conjunction with current knowledge of historic Sub-Saharan habitat dynamics to investigate patterns and timings of diversification within the genus *Sylvietta*. Finally, we briefly explore the phylogeographic diversity of two widespread members of this genus, *S. virens* and *S. rufescens*, in relation to phylogeographic diversification in lowland forests and arid-land habitats, respectively.

II.2 Methods

II.2.1 Taxon Sampling and Molecular Data

We gathered 51 total samples of *Sylvietta* from American museum collections (*S. virens* = 14, *S. rufescens* = 14, *S. whytii* = 9, *S. brachyura* = 3, *S. leucophrys* = 3, *S. denti* = 2, *S. ruficapilla* = 2, *S. isabellina* = 3, *S. philippae* = 1). Five outgroups were chosen

based on varying degrees of relation to *Sylvietta* (Alström *et al*. 2006; Fregin *et al*. 2012) and availability either as fresh tissue or GenBank records: three members of Sylviidae (*Hylia prasina*, *Sylvia subcaeruleum*, *Eremomela usticollis*) and two members of Cisticolidae (*Camaroptera brachyura* and *Apalis thoracica*). Whole genomic DNA was extracted from all fresh tissue samples (N = 46) using standard proteinase K digestion according the manufacturer's instructions (DNeasy). Five samples (*S. isabellina* = 3, *S. denti* = 1, *S. philippae* = 1) were only available as toe pad cuttings and an extended extraction method was utilized to isolate DNA from these samples. Toe pad clips were soaked in a 1X Phosphate-buffered saline (PBS) solution for 72 hours and then broken up using sterile blades. These samples then underwent a 24 hour, 56 degree Celsius heat bath with proteinase K added every six hours, followed by standard (DNeasy) extraction methods.

Polymerase chain reactions (PCR) were employed to amplify two mitochondrial (mtDNA) loci in all 51 samples: NADH-dehydrogenase-2 (ND2) and cytochrome oxidase *b* (CYTB). A sub-set of individuals (N = 30; all representing fresh tissue samples) were chosen from preliminary mtDNA phylogenies for amplification of two nuclear (nuDNA) loci: nuclear Transforming growth factor, beta 2 (TGF β2) and Myoglobin intron-2 (MYO). Standard primers were utilized to amplify all four loci. PCR protocols consisted of a "hot start" of 94° C for 4 minutes, denaturing at 95° C for 45 seconds, annealing between 50-60° C for 30 seconds, an extension step of 72° C for one minute and a final extension of 72° C for 10 minutes. Single-pass Sanger sequencing, using the same primer sets as utilized for PCR, was performed using ABI

Big Dye Terminator v3.1 at the Beckman-Coulter Genomics facility (Danvers, MA). Both mtDNA and nuDNA loci were aligned in Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI) and both mtDNA markers were assessed for errant stop codons and verified to be protein-coding.

II.2.2 Phylogenetic Analysis and Divergence Estimates

Two concatenated data sets were utilized for phylogenetic analysis: 1) a mtDNA-only data set with all sampled individuals (N = 51) and, 2) a smaller sub-set (N = 30) consisting of all four loci (mtDNA + nuDNA). We used PartitionFinder (Lanfear *et al*. 2014) to find the best fit models of evolution and appropriate partitioning schemes for both concatenated data sets. Bayesian inference was then undertaken using MrBayes 3.2.5 (Huelsenbeck and Ronquist 2001) with two runs of eight Markov chain Monte Carlo (MCMC) chains of 10 million generations sampled every 1000 generations. Tracer v1.6 (Rambaut *et al*. 2014) was used to visualize post-run statistics and trace in order to determine if the runs had reached stationarity and to determine an appropriate burn-in. Additionally, genetic distance measures in the form of average uncorrected *p*-distances for the ND2 data between clades recovered from the mtDNA Bayesian analysis, were calculated using Mega6 (Tamura *et al*. 2013).

BEAST v2.3.1 (Drummond *et al*. 2012) was utilized on the four-locus dataset (N=30) to derive a species tree (*BEAST template) and divergence estimates (standard BEAST template). Excluded from this dataset were *S. philippae* and *S. isabellina*, for which we could not amplify any length of either nuclear loci, therefore divergence

estimates for these taxa relative to their sister taxa were estimated from mtDNA only. For the four-locus dataset, a relaxed, lognormal clock was chosen to estimate divergence times, utilizing lineage substitution rates (per lineage/million years) gathered from previous research publications. Substitutions rates for three of the four genes were taken from Lerner *et al*. (2011) (ND2 = 0.029, CYTB = 0.014, TGF β2 = 0.017) while the MYO substitution rate (= 0.002) was taken from Voelker *et al*. (2015). Standard deviations (SD) for both mitochondrial genes and TGF β2 were (CYTB = 0.001, ND2 = 0.0025, TGF β2 = 0.002) were taken from Lerner *et al*. (2011) and a standard 0.0002 was used for MYO (Voelker *et al*. 2015). Both BEAST analyses (*BEAST and standard BEAST) utilized a normal Yule process speciation prior and linear population function (coalescent model prior) in two MCMC runs of 100,000,000 generations sampled every 1,000 generations. Tracer v1.6 was used to assess that proper parameter mixing had been achieved (all ESS > 200) and to assess an appropriate burn-in (25%). We used LogCombiner v2.3 (Drummond *et al*. 2012) to initiate the burn-in and combine both BEAST runs. The combined tree topologies were analyzed using TreeAnnotator v2.3 (Drummond *et al*. 2012).

II.2.3 Historical Biogeography

Ancestral range estimation was performed using the BioGeoBEARS package in the R statistical program (Matzke 2013a,b). We made use of BioGeoBEARS ability to run two basic models of ancestral area reconstruction, the DEC and DEC + J models. The DEC (dispersal-extinction cladogenesis) model utilizes two parameters: the rate of

dispersal (range expansion) and the rate of extinction (range contraction), while fixing the cladogenesis model. The second model DEC + J, uses the same parameters as the DEC model while adding a third free parameter (J) corresponding to long distance dispersal. This free parameter allows one daughter lineage to move to a non-adjacent area outside of the ancestral range. We were then able to compare the two models (DEC vs. DEC + J) using a likelihood ratio test (LRT).

We coded each species as being present or absent in the following five sub-regions using distributional maps found in Bairlein (2006): Congo, Sudanian, Zambezian, Ethiopian + Somalian, and Southern Africa. These sub-regions follow Linder *et al*. (2012), where sub-regions for birds were statistically defined using cluster analysis. We also delineated a sixth sub-region, Afro-montane, which has traditionally been recognized as a unique bioregion (White 1983). Despite not being defined as statistically unique by Linder *et al*. (2012), we included this region as a separate biogeographic area to more finely understand the biogeographic history of *S. leucophrys*, an Afro-montane endemic. Additionally Linder *et al*. (2012) determined Benin to be part of the arid Sudanian bioregion despite the current existence of Afro-tropical forest patches. In order to keep the Sudanian designation from over complicating the biogeographical history of *S. virens*, a lowland forest endemic whose range includes Beninoise forest patches, we excluded the Sudanian bioregion for this species and instead designated it as solely inhabiting the Congolian bioregion.

II.3 Results

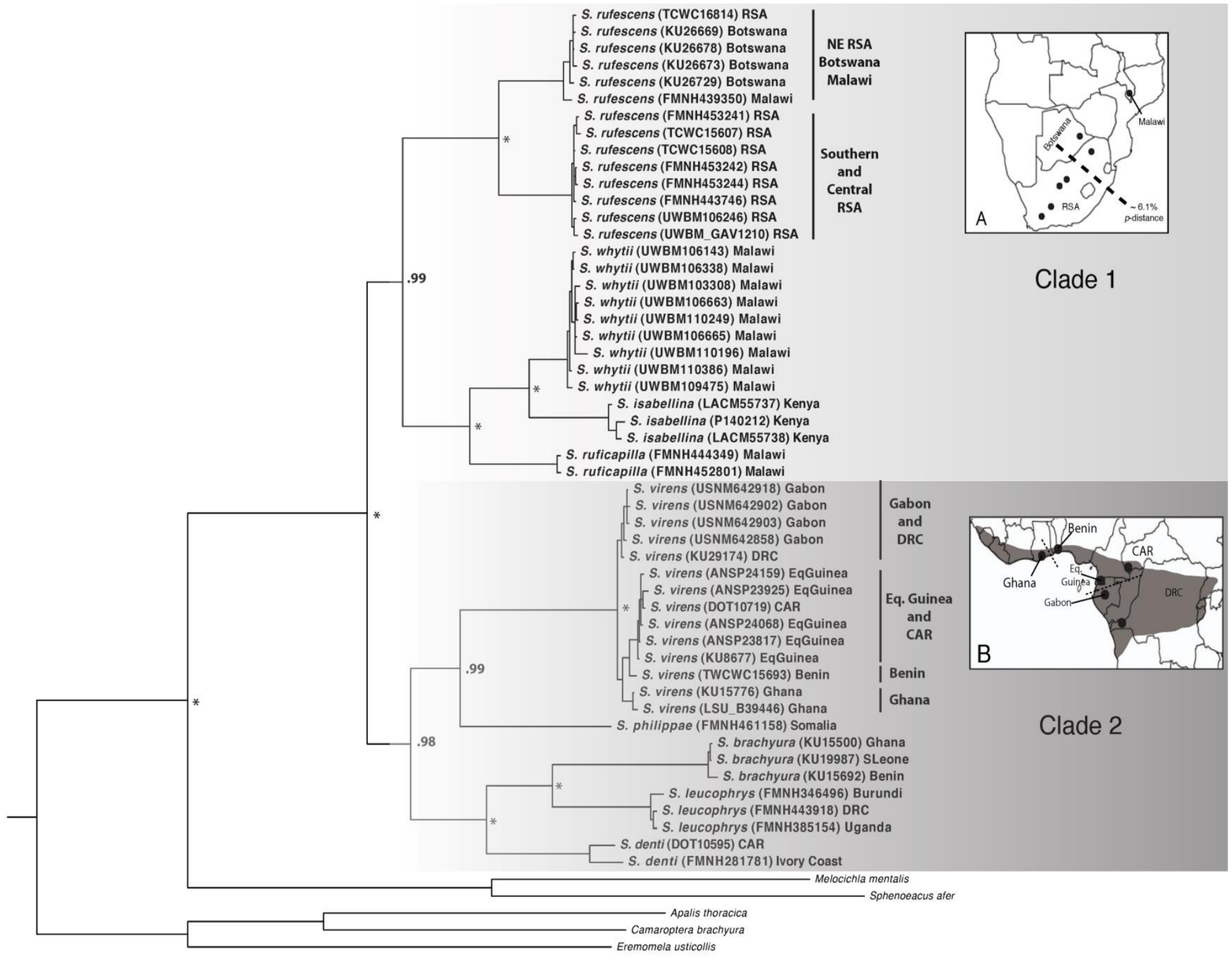
II.3.1 Taxon Sampling and Molecular Data

We were able to obtain 2,060 base pairs (bp) of mtDNA (ND2 = 1041bp, CYTB = 1018bp) from 46 of the 51 individuals sampled. Presumably due to the poor quality of the five toe pad samples (*S. isabellina* = 3, *S. denti* = 1, *S. philippae* = 1), we were only able to sequence fragments of each mitochondrial gene for these samples (ND2 = 307-1001bp, CYTB = 641-1005 bp). For the nuclear loci, we recovered a total of 1,317 base pairs from 30 individuals (MYO = 685bp, TGF β2 = 632bp). Again, due to the poor quality of the five toe pad samples, we were unable to recover any fragments of either nuclear locus.

II.3.2 Phylogenetic Analysis

Using MrBayes, we recovered the first strongly supported, multi-locus trees for the genus *Sylvietta*. The results of both Bayesian analyses (mtDNA-only and four-gene) produced strongly supported trees (≥ 98% posterior probability (PP)) with congruent overall topologies with the four-gene phylogram missing *S. isabellina* and *S. philippae.* (Figs. 2 and 3). Both analyses recovered two major clades: Clade 1, corresponding to individuals from the Zambezian, Southern African, and Ethiopian-Somalian sub-regions and Clade 2, corresponding to the Congo, Afro-montane, Sudanian, and Ethiopian-Somalian sub-regions (Figs. 2 and 3).

Figure 2. Bayesian molecular phylogeny of the genus *Sylvietta* utilizing only the mitochondrial dataset (CYTB and ND2). Values close to the nodes represent posterior probabilities (* = posterior probability of 1.0). Inset A: the sampling of *S. rufescens* and general line of division between the two divergent haplogroups. Inset B: the general sampling of *S. virens* in relation to haplogroup structuring.


S. rufescens (TCWC16814) RSA
S. rufescens (KU26669) Botswana
S. rufescens (KU26678) Botswana
S. rufescens (KU26673) Botswana
S. rufescens (KU26729) Botswana
S. rufescens (FMNH439350) Malawi
NE RSA Botswana Malawi
S. rufescens (FMNH453241) RSA
S. rufescens (TCWC15607) RSA
S. rufescens (TCWC15608) RSA
S. rufescens (FMNH453242) RSA
S. rufescens (FMNH453244) RSA
S. rufescens (FMNH443746) RSA
S. rufescens (UWBM106246) RSA
S. rufescens (UWBM_GAV1210) RSA
Southern and Central RSA
S. whytii (UWBM106143) Malawi
S. whytii (UWBM106338) Malawi
S. whytii (UWBM103308) Malawi
S. whytii (UWBM106663) Malawi
S. whytii (UWBM110249) Malawi
S. whytii (UWBM106665) Malawi
S. whytii (UWBM110196) Malawi
S. whytii (UWBM110386) Malawi
S. whytii (UWBM109475) Malawi
S. isabellina (LACM55737) Kenya
S. isabellina (P140212) Kenya
S. isabellina (LACM55738) Kenya
S. ruficapilla (FMNH444349) Malawi
S. ruficapilla (FMNH452801) Malawi
S. virens (USNM642918) Gabon
S. virens (USNM642902) Gabon
S. virens (USNM642903) Gabon
S. virens (USNM642858) Gabon
S. virens (KU29174) DRC
Gabon and DRC
S. virens (ANSP24159) EqGuinea
S. virens (ANSP23925) EqGuinea
S. virens (DOT10719) CAR
S. virens (ANSP24068) EqGuinea
S. virens (ANSP23817) EqGuinea
S. virens (KU8677) EqGuinea
Eq. Guinea and CAR
S. virens (TWCWC15693) Benin
Benin
S. virens (KU15776) Ghana
S. virens (LSU_B39446) Ghana
Ghana
S. philippae (FMNH461158) Somalia
S. brachyura (KU15500) Ghana
S. brachyura (KU19987) SLeone
S. brachyura (KU15692) Benin
S. leucophrys (FMNH346496) Burundi
S. leucophrys (FMNH443918) DRC
S. leucophrys (FMNH385154) Uganda
S. denti (DOT10595) CAR
S. denti (FMNH281781) Ivory Coast
Melocichla mentalis
Sphenoeacus afer
Apalis thoracica
Camaroptera brachyura
Eremomela usticollis
.99
.98
*
Malawi
Botswana
RSA
~ 6.1% p-distance
A
Clade 1
Benin
CAR
Ghana
Eq. Guinea
Gabon
DRC
B
Clade 2

Across these two groups, the mitochondrial-only analysis recovered eight clades (plus the one individual of *S. philippae*), reflecting the currently recognized nine species of *Sylvietta* (Fig. 2). Clade 1, *S. rufescens* + *S. whytii* + *S. isabellina* + *S. ruficapilla*, recovers *S. rufescens* as sister to the remaining three species, with *S. ruficapilla* as sister to *S. whytii* + *S. isabellina* (Fig. 2). Clade 2, *S. virens* + *S. philippae* + *S. brachyura* + *S. leucophrys* + *S. denti*, recovers *S. virens* and *S. philippae* as sister species to the remaining three species, with *S. denti* sister to *S. brachyura* and *S. leucophrys*. Both the mtDNA-only and combined analysis show little intra-specific genetic variation within *Sylvietta*, with the exception of shallow diversification within *S. virens* and a substantial genetic divergence within *S. rufescens*. *S. virens* displays a pattern of shallow geographic structuring consisting of sub-clades from Gabon, Equatorial Guinea + the Central African Republic (CAR), Ghana, and additionally, two individuals from Benin and the Democratic Republic of the Congo (Fig 2). These sub-clades are characterized by low levels of genetic divergence, with an average uncorrected *p*-value across the group of ~1.6 %. Conversely, *Sylvietta rufescens* is divided into two strongly supported sub-clades, one consisting of individuals from central and southern South Africa (RSA) and the other made up of individuals from Botswana and northern South Africa (plus one Malawi individual), separated by an uncorrected *p*-distance of 6.1% (Figs. 2 and 3).

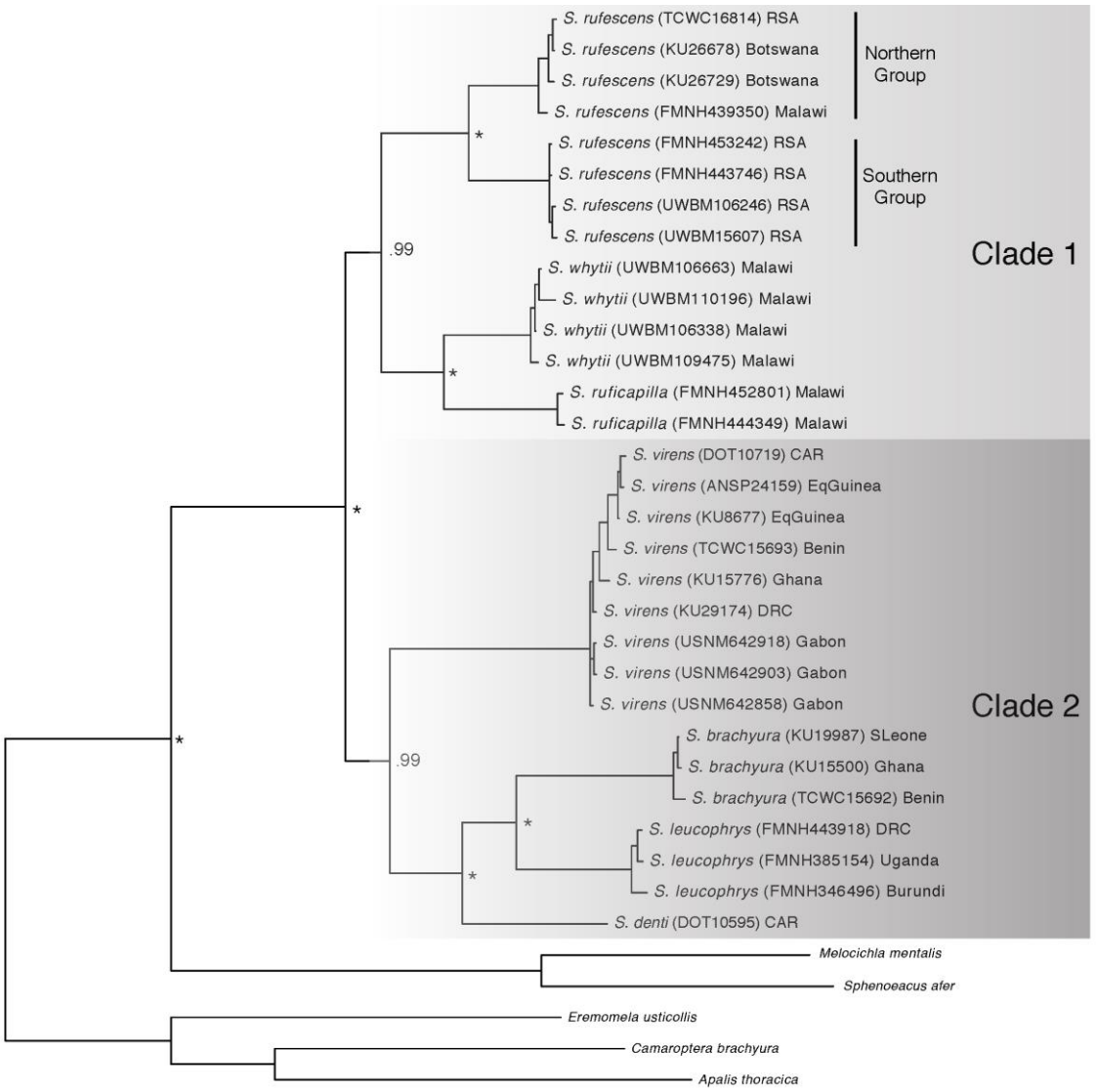


Figure 3. Bayesian molecular phylogeny of the genus *Sylvietta* using all five loci (two mitochondrial and two nuclear). Values close to the nodes represent posterior probabilities (* = posterior probability of 1.0)

II.3.3 Species Tree and Divergence Estimates

The inability to amplify either nuclear loci (TGF β2 or MYO) for toe pad samples of *S. isabellina* and *S. philippae* led to their obvious exclusion from the BEAST analyses. However, to gain rough divergence estimates for these two species (*S. isabellina* and *S. philippae*) we used the uncorrected p-distance for CYTB from their sister taxa to calculate general divergence times. For taxa included in the BEAST analysis, both the BEAST and *BEAST analyses recovered topologies congruent with one another as well as with the Bayesian analyses (Fig. 4). With the exception of the poor support (PP = 0.43) at the node splitting *S. virens* from the other three taxa in this clade, support for both trees was strong (PP = 1.0). The molecular clock estimates the initial divergence in the genus as being in the late Miocene (~5.6 Ma). With the exception of a late Miocene origin for *S. virens* (~ 5.4 Ma) and a putative Pleistocene origin (~ 1.43 Ma, based on CYTB) for *S. whytii* and *S. isabellina*, we recover Pliocene ages (5-1.8 Ma) for the remaining origins of all species within the genus (Fig. 4). The strongly supported sub-clades of *S. rufescens* are estimated to have diverged in the mid Pleistocene (~ 1.7 Ma).

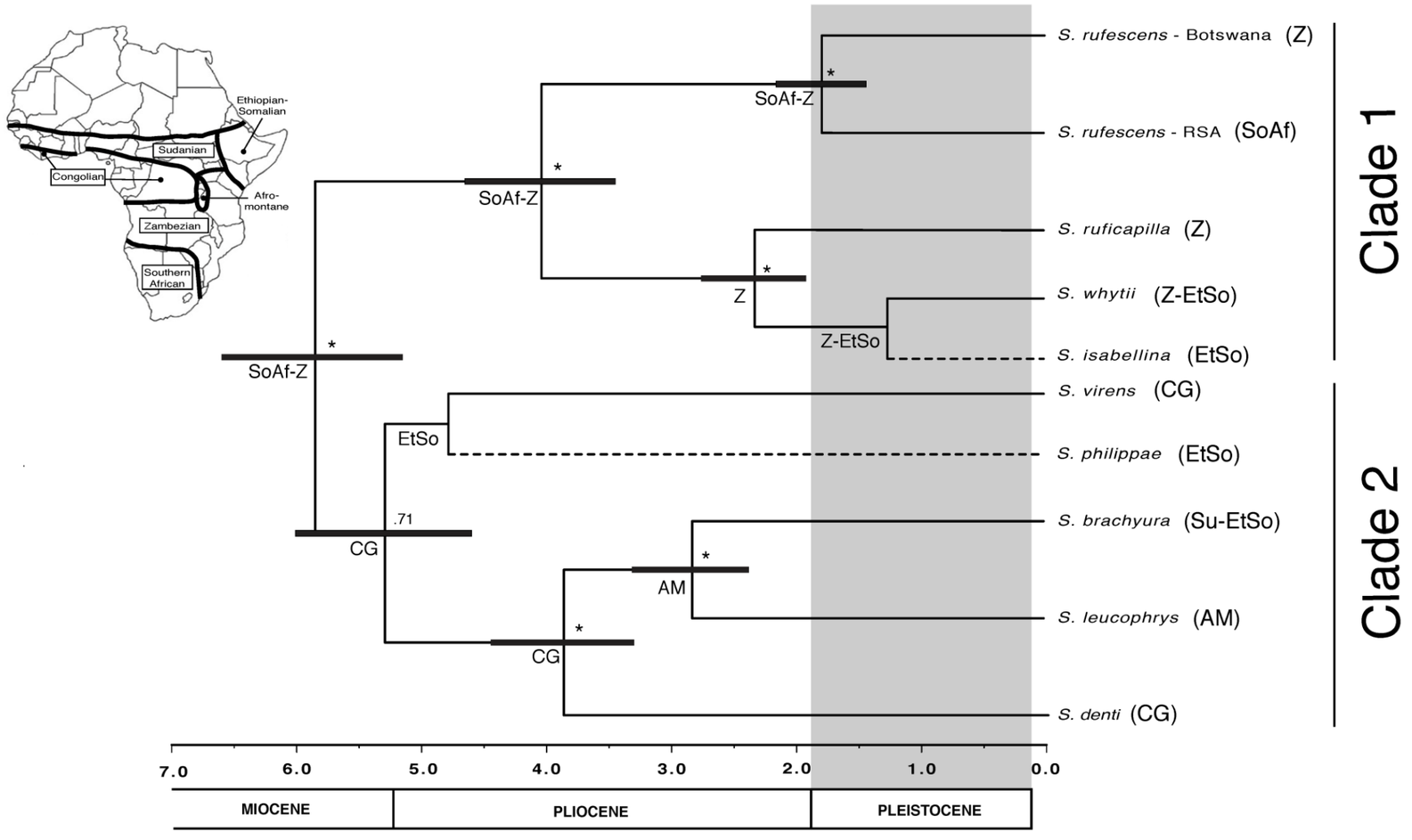


Figure 4. Species tree from *BEAST with molecular clock estimates of lineage divergence dates for the genus *Sylvietta*, based on evolutionary rates from Lerner *et al*. 2011 (CYTB, ND2, TGF β2) and Voelker *et al*. 2016 (MYO). Nodal values are posterior probabilities (* = 1.0) and nodal bars represent the 95% highest posterior density intervals. Dashed branches represent divergence estimates from mtDNA only. BioGeoBEARS ancestral range estimations are represented at each node, with the areas defined as follows: SoAf = Southern Africa; Z = Zambezian; CG = Congolian; AM = Afro-montane; Su = Sudanian; EtSo = Ethiopian-Somalian. Individual species distributions using these abbreviations follow each species name.

II.3.4 Ancestral Area Estimates

The DEC + J model in BioGeoBEARS was shown to be significantly better (-ln 22.75; $P = .005$) than the simple DEC model. Overall, the ancestral area estimate suggests a roughly southern origin (South African–Zambezian) for the basal node in *Sylvietta*, representing the divergence of Clade 1 from Clade 2 (Fig. 4). For Clade 1, a southern to northern diversification pattern through the arid habitats of eastern Africa is estimated. On the other hand, Clade 2 is estimated to have a Congo Forest origin, with subsequent eastward colonization into the Afro-montane forests and Ethiopia-Somalia. For *S. virens* we observe a potential back colonization of the Congo Forest from Ethiopia-Somalia, while *S. brachyura* ultimately colonizes the Sudanian region sometime after dispersing into Ethiopia-Somalia.

II.4 Discussion

II.4.1 Systematics

This is the first complete investigation of species relationships within *Sylvietta* and therefore represents the first view of the genus as represented by molecular markers. Bairlein (2006) reported that two superspecies groups are found within *Sylvietta*: 1) *S. brachyura* + *S. whytii* + *S. philippae* and 2) *S. rufescens* + *S. isabellina*. The determination of these groups appears to be based, at least in part, on morphology, geographical range, and habitat requirements. Yet the results of both Bayesian analyses (mtDNA only and all genes combined) fail to recover any of the aforementioned superspecies taxa as being sister to one another. Instead, both analyses recover a

completely novel set of relationships for all members of *Sylvietta* (Figs. 2 and 3). First, our analyses indicate that the genus can be primarily grouped into two major clades: Clade 1 consists of four species (*S. rufescens* + *S. whytii* + *S. isabellina* + *S. ruficapilla*) while Clade 2 is made up of the remaining five species (*S. virens* + *S. philippae* + *S. brachyura* + *S. leucophrys* + *S. denti*) (Figs. 2 and 3).

In Clade 1, *S. rufescens* is recovered as sister to the remaining species of this group and not as sister to *S. isabellina* as previously suggested by Bairlein (2006). *S. isabellina* is recovered as sister to *S. whytii*, which despite being substantially different in plumage (see Sinclair and Ryan 2010) do display sympatric ranges (Fig. 1). In fact, the species relationships in Clade 1 are reinforced strongly by range and habitat data, as all members of this group favor arid habitats (Bairlein 2006). Additionally, and contrary to Irwin (1968), we do not recover *S. whytii* as sister to *S. ruficapilla*.

Clade 2 is recovered in both analyses with a *S. virens* + *S. philippae* sister relationship, with those two species as sister to the remaining three species (*S. brachyura* + *S. leucophrys* + *S. denti*) (Figs. 2 and 3). Despite the hypothesis that *S. brachyura* forms a superspecies with *S. whytii* and *S. philippae* (Bairlein 2006), we instead recover *S. brachyura* as sister to *S. leucophrys*. The phylogenetic relationships in this group correspond with geographic range data, as all members of this clade exist in the northern and central regions of Sub-Saharan Africa.

II.4.2 Historical Biogeography and Phylogeography

We estimate a southern origin for *Sylvietta* (South African-Zambezian) with initial diversification in the genus taking place ~ 5.6 Ma (Late Miocene), splitting the genus into two major clades (Fig. 4). This date roughly corresponds to the initiation of an inter-glacial phase in the African climate which led to significant lowland forest expansion between 5-3.5 Ma (Hamilton and Taylor 1991; Cane and Molnar 2001; Feakins *et al*. 2005; Sepulchre *et al*. 2006); a period which has been linked to divergences in other avian assemblages (Voelker *et al*. 2014; Voelker *et al*. 2015).

Clade 1 consists of four arid-adapted species (Fig. 2 and 3) and our ancestral area estimations place the origin of this group in southern Africa (South African-Zambezian) with diversification beginning ~3.6 Ma, with *S. rufescens* the first lineage to diverge (Fig. 4). The remaining three species (*S. ruficapilla* + *S. isabellina* + *S. whytii*) are estimated to share a common ancestor of Zambezian origin with diversification of this group starting ~2.14 Ma. This date generally coincides with the retraction of Afro-tropical forests westward from East Africa as a result of increased global aridity, a scenario which has been linked to diversification of several avian lineages (Outlaw *et al*. 2007; Voelker and Outlaw 2008; Voelker *et al*. 2010; Fuchs *et al*. 2011; Voelker *et al*. 2015). The westward retraction of African forests and corresponding expansion of arid habitat would have opened a corridor in eastern Africa connecting northern and southern arid zones. This scenario may explain diversification of the remaining species of this clade, as colonization from the Zambezian sub-region into Ethiopia and Somalia would have become possible from this point forward, by arid-adapted species. The southern to

northern colonization and diversification in Clade 1 represents a rare pattern for African avian species, which has only been demonstrated in two other investigations thus far (Voelker *et al*. 2014; Voelker *et al*. 2015).

Clade 2 consists of three lowland forest endemics and two arid-adapted species, and the BioGeoBEARS analysis estimates a west to east colonization pattern for this group, likely as a result of eastward forest expansion as discussed above (Fig. 4). The Congo Forest is estimated as the basal distributional area for this clade (Late Miocene: ~5.4 Ma. The ancestor of *S. virens* + *S. philippae* is estimated to have an Ethiopian-Somalian origin, suggesting the colonization of northeastern Africa as forests expanded eastward (~ 4.83 Ma). Further, our ancestral area estimation reconstructs a Congo Forest origin for the ancestor of *S. brachyura* + *S. leucophrys* + *S. denti* (~3.8 Ma). Subsequent colonization of the Afro-montane region by the ancestor of *S. brachyura* and *S. leucophrys* (~2.8 Ma) indicates eastward colonization from lowland forests between 3.8 and 2.8 Ma (Fig. 4). Forest contraction likely explains the later stage of diversifications in this clade. *Sylvietta philippae*, which was estimated as having diverged during forest expansion, seemingly shifted to arid habitats as a consequence of westward forest contraction and fragmentation during this period. Additionally, the divergence of *S. leucophrys* and *S. brachyura* is estimated around 2.8 Ma as forest habitat contracted westward, leading to a habitat shift for *S. brachyura* into arid habitats with subsequent diversification into the Sudanian bioregion. Overall for Clade 2, we estimate a general pattern of early eastward colonization followed by diversification during subsequent forest retraction, with several forest lineages adapting to arid habitats. This scenario is in

line with investigations showing the impact of Pliocene forest expansion (Voelker *et al*. 2014; Voelker *et al*. 2016) and subsequent Plio-Pleistocene retraction and fragmentation (Outlaw *et al*., 2007; Fjeldså and Bowie 2008; Voelker and Outlaw 2008; Voelker *et al*., 2010, 2012; Fuchs *et al*., 2011) in creating avian diversity through complex patterns of habitat dynamics.

II.4.3 Intra-specific Diversification in Sylvietta virens

Several recent studies of avian lowland forest species have demonstrated the existence of complex patterns of geographic structure among deeply divergent haplotypes from Sierra Leone to the eastern Congo forest (*Illadopsis*: Nguembock *et al*. 2009; *Hylia prasina*: Marks 2010; *Sheppardia*: Voelker *et al*. 2010; *Bleda*: Huntley and Voelker 2016). These diversification patterns indicate multiple climate-driven vicariance events throughout the Plio-Pleistocene, in which lineages were isolated in forest refuges. This pattern is not restricted to avian species, as recent evidence of similar patterns have been demonstrated in several mammalian taxa (*Sylvisorex*: Querouil *et al*. 2003; *Lemniscomys striatus*: Nicolas *et al*. 2008; *Praomys*: Bryja *et al*. 2010 and Nicolas *et al*. 2011; *Crocidura olivieri*: Jacquet *et al*. 2015). *Sylvietta virens*, a widespread lowland forest endemic, displays similar phylogeographic patterns with those mentioned above (Figs. 2 and3). Yet unlike the deeply divergent geographically genetic structuring demonstrated in those studies, *S. virens* is characterized by geographic structuring with relatively shallow genetic divergence (average within group ND2 *p*-distance of ~1.6%) (Figs. 2 and 3). We recover sub-clades corresponding to

Gabon, Equatorial Guinea + CAR, and Ghana, as well as limited evidence for the divergence of Benin and the DRC populations. This pattern is not particularly surprising in light of other investigations which found limited divergence between forest populations of avian (Olive Sunbird: Bowie *et al*. 2004; Wattle-eyes: Njabo *et al*. 2008) and mammalian taxa (chimpanzees: Gonder *et al*. 2011; Myonycterini bats: Nesi *et al*. 2013). Bowie (*et al*. 2004) contended that a lack of divergence between Olive Sunbird haplotypes could be due to the species' use of a wide range of forest habitats, potentially making it a more capable disperser. Similarly, *S. virens* also inhabits a wide range of habitats (primarily forest edges and clearings of primary forests, but also secondary and gallery forest, bush, woodland and mangrove edges, as well as moist savannah thickets) (Bairlein 2006), suggesting a higher potential for dispersal which may explain the lack of deep genetic divergences within this species (Fig. 2). Overall, when biogeographic investigations of Afro-tropical forest taxa are taken as a whole, the patterns demonstrate that the life history of each organism allowed for different responses to shared historical pressures.

II.4.4 Intra-specific Diversification in Sylvietta rufescens

The recovery of two sub-clades in *S. rufescens* corresponding to a "northern" group (Malawi, Botswana and northeast South Africa) and a "southern" group (southern and central South Africa) with ~6% uncorrected pairwise distance between them is difficult to explain, yet not entirely surprising. While the majority of biogeographic investigations of arid-adapted taxa have focused on analyzing the genetic structuring of

northern versus southern African populations in relation to Afro-tropical forest dynamics (Lorenzen *et al*. 2012; Voelker *et al*. 2014; Voelker *et al*. 2015), several studies scrutinizing potential regional diversification within southern Africa have been performed. Results suggest that several avian species within this region have demonstrated that historic habitat variation may have led to genetic divergences across ecotones (Ryan *et al*. 1998; Ryan and Bloomer 1999; Outlaw *et al*. 2007; Ribeiro *et al*. 2011; Oatley *et al*. 2012; Voelker *et al*. 2014). Additionally, a phylogeographic study of the Fiscal Shrike (*Lanius collaris*) found surprising levels of genetic variation between populations within Mozambique, where populations distributed from northern Mozambique northward and very different from populations distributed from southern Mozambique southward. Yet, Mozambique is far south of the east African suture zone shown in other avian taxa (Fuchs *et al*. 2011). Finally, many mammalian and reptilian taxa display complex patterns of genetic variation in southern Africa driven by isolation in stable arid refugia during inter-glacial cycles, in combination with disruptive barriers (i.e. rivers and massive sand flows) (mammals: Smit *et al*. 2007; Smit *et al*. 2010; Lorenzen *et al*. 2012; reptiles: Matthee and Fleming 2002; Tolley *et al*. 2010; Barlow *et al*. 2013).

Given the lack of any obvious barriers (e.g. rivers, mountain chains) between the two divergent haplogroups in *S. rufescens*, in addition to the fact that both populations share the same habitat type, it seems probable that this pattern is the result of historical isolation in arid refugia during the Pleistocene (~1.62 Ma). However, the small number of individuals sampled in the present study makes only speculation possible and

increased sampling within *S. rufescens* range will be necessary for a greater understanding of this pattern.

II.4.5 Conclusions

The inter- and intra-specific patterns and timing of diversification events within the genus *Sylvietta* highlights the complex historical impact of climate-induced habitat cycling on Sub-Saharan African species. The expansion and contraction of Afro-tropical forests, particularly 5-3 Ma, have undoubtedly played an important role in facilitating the colonization of eastern habitats and subsequent diversification events within this genus. The southern to northern colonization and diversification pattern displayed by several members of *Sylvietta* is particularly interesting as it is only the third instance of this pattern demonstrated in Afro-avian species (Voelker *et al*. 2014; Voelker *et al*. 2015). Additionally, the diversification patterns demonstrated in *S. rufescens* and *S. virens* serve to reflect two major points of interest. First, the novel divergence between members of *S. rufescens* highlights the lack of understanding regarding cryptic regional diversity in arid-adapted species, a consequence of incomplete sampling of avian species in this bioregion. Given the pattern recovered in this study, in conjunction with those previously cited, it seems likely that diversity in southern Africa has been underestimated, highlighting the substantial need for further sampling across this region. Second, the lack of deep divergences within *S. virens* in the face several recent investigations recovering deeply geographically-structured genetic haplogroups across lowland forests highlights the importance of increased collection in this region to fully

understand the complex interplay between climate-driven habitat fragmentation and connectivity of populations.

CHAPTER III

CRYPTIC DIVERSITY IN AFRO-TROPICAL LOWLAND FORESTS: THE SYSTEMATICS AND BIOGEOGRAPHY OF THE AVIAN GENUS *BLEDA* (BRISTLE-BILLS)[*]

III.1 Introduction

Early investigations into the distributional patterns of Afro-tropical avifauna revealed a complex mosaic within both lowland and montane forest regions (Diamond and Hamilton 1980; Crowe and Crowe 1982). Among the patterns described from these large, descriptive biogeographic analyses were regions with high numbers of endemic species (e.g., the eastern Congo Basin, Eastern Arc and Albertine Rift Mountains, and Cameroon highlands). Due to the lack of obvious geographic barriers between these areas that could explain geographic variation within and among species, researchers concluded that they were the sites of ancient forest refuges and invoked the Pleistocene Forest Refuge Hypothesis (PFRH, where the upper boundary was c. 1.8 Ma), first proposed to describe similar patterns in South America (Haffer 1974), as an isolating mechanism (Mayr and O'Hara 1986; Prigogine 1988). The PFRH posits that global climate oscillations led to widespread fragmentation and coalescence cycles within Afro-tropical forests during the Pleistocene and these forest fragmentation events subsequently led to the allopatric divergence of formerly widespread forest-dwelling

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populations. The hypothesis that forest diversity was derived from ancient forest refuges led to an early research emphasis on endemic taxa as well as those with seemingly disjunct range distributions.

In contrast to areas rich in endemic species, the recognition of many widespread, morphologically monotypic species across the Guineo-Congolian lowland forests (Fig. 1) in conjunction with the proliferation of modern molecular divergence estimates led to the Montane Speciation Hypothesis (Roy 1997; Fjeldså and Lovett 1997; Roy *et al.* 2001; Fjeldså *et al.* 2005; Fjeldså *et al.* 2007; Fjeldså and Bowie 2008). A pattern emerged in these investigations in which the ages of Afro-tropical montane avifauna were revealed to be relatively young (Miocene and later), while lowland forest lineages were found to be "ancient" (ca. 12 - 20 Ma). The young ages of montane divergence events led to a focus on montane forest regions as centers of diversification. The ancient ages of most lowland forest species, combined with the observation that many lineages found there lack plumage variation across their ranges, led to the conclusion that Guineo-Congolian lowland forests, with the exception of a few lineages, were relatively unimportant areas for creating diversity. As a result, the lowland forests became relegated to the role of "evolutionary museum", where most species have (presumably) persisted essentially unchanged since the beginning of the Miocene epoch (c. 5 Ma – 23 Ma) (Fjeldså and Lovett 1997).

As a consequence, the PFRH and "evolutionary museum" idea of the Montane Speciation Hypothesis resulted in the view that lowland forest dwelling avian species with widespread ranges and lack of plumage variation were biogeographically

uninformative. Mayr and O'Hara (1986) went as far as to publish a list of 107 taxa deemed uninformative based on their lack of plumage variation (equating lack of variation with lack of diversification). Yet despite the continued prevalence of the "evolutionary museum" concept, several recent efforts have revealed a different evolutionary pattern for avian species within lowland forests. For example, the widespread, monotypic Green Hylia (*Hylia prasina*) was shown to possess highly discrete geographic structure and deep genetic divergences between major lowland forest blocks (Marks 2010). The systematics and phylogeography of the forest robin genus *Stiphrornis* has been analyzed no less than three times, with each investigation revealing deep-rooted cryptic diversity and new species (Beresford and Cracraft 1999; Schmidt *et al.* 2008; Voelker *et al.* 2016). In addition, the timing of diversification leading to geographic structuring within several recently studied lowland taxa has proven to be relatively recent (within the last 1.8 my), a result that seems to suggest the possibility of the Pleistocene Forest Refuge Hypothesis as a potential explanation for at least some diversification patterns (for birds: Voelker *et al.* 2013; Fuchs and Bowie 2015; for mammals: Quérouil *et al.* 2003; Nicolas *et al.* 2008). Yet, not every widespread lowland species harbors phylogeographic structure within its range, as was demonstrated for the Olive Sunbird (*Nectarinia olivacea*) (Bowie *et al.* 2004a). Nor does every species display patterns of Pleistocene-aged diversification from close relatives; some divergences date to the Pliocene (e.g., Njabo *et al.* 2008; Voelker *et al.* 2010a).

As investigations of distributional patterns of lowland taxa have increased, so has the understanding of historical and current mechanisms within forest blocks for disrupting gene flow and generating diversity. A recent comparative study revealed genetic diversification in four out of ten avian species distributed on either side of the Congo River (Voelker *et al.* 2013), providing support for the Riverine Barrier Hypothesis (RBH) (Wallace 1852). Indeed, although originally developed from distributional observations of Amazonian taxa, the RBH has gained increasing support in Africa, with several vertebrate lineages displaying varying levels of diversification across the Congo, Niger, Sanaga or Ogooue river systems (for rodents: Kennis *et al.* 2011; Nicolas *et al.* 2012; Bohoussou *et al.* 2015; Jacquet *et al.* 2015; for bats: Hassanin *et al.* 2015; for birds: Voelker *et al.* 2013; Fuchs and Bowie 2015).

One avian species displaying a substantial level of diversification across the Congo River was the Red-tailed Bristle-bill (*Bleda syndactylus*) (Voelker *et al.*, 2013). The result was surprising due to a lack of plumage variation, which originally caused this species to be included on the aforementioned list of uninformative lowland forest taxa generated by Mayr and O'Hara (1986). The *Bleda* Bristle-bills are comprised of four understory dwelling species, all of which are endemic to the lowland forests of Africa (Fig. 1) and with the exception of one species, lack plumage variation across their range. The Red-tailed Bristle-bill (*B. syndactylus*) inhabits a wide range encompassing all Guineo-Congolian lowland forests, while the Green-tailed Bristle-bill (*B. eximius*) and the Grey-headed Bristle-bill (*B. canicapillus*) are West African endemics (Fig. 5). The Yellow-lored Bristle-bill (*B. notatus*) and its sub-species the Yellow-eyed Bristle-

bill (*Bleda notatus ugandae*) are endemic to parts of the Lower Guinean and Congo Forests (see Fig. 5). The Yellow-eyed Bristle-bill (*B. ugandae*) was also included in Mayr and O'Hara's (1986) list of uninformative taxa, but unlike *B. syndactylus*, it did not display genetic differentiation across the Congo River (Voelker *et al.* 2013). A lack of diversification in the former species was surprising given that it, like the Red-tailed Bristle-bill, is an understory specialist. Understory specialists are generally less vagile species and should hypothetically be more susceptible to diversification from forest fragmentation events. Overall, the disparate diversification patterns found in a relatively small geographic region provides an impetus for investigating patterns within the genus *Bleda.*

To date, just two investigations of species limits and relationships within the genus *Bleda* exist. Chappuis and Erard (1993) examined the systematics of *Bleda* using a small dataset of morphological and behavioral (song) characters. Their study recognized four species (*syndactylus, canicapillus, eximius* and *notatus*) and placed *B. ugandae* as subspecies of *B. notatus.* Beresford (2002) examined the phylogenetics of *Bleda* using a molecular dataset composed of 96 specimens and two loci. Her research confirmed the four species recognized by Chappuis and Erard (1993), but also suggested the possibility of *B. n. ugandae* being a distinct species. In addition, based on observed differences within the mitochondrial dataset of *B. syndactylus*, Beresford (2002) speculated that that species might be composed of multiple taxa; her limited dataset did not allow for additional assessments of that possibility.

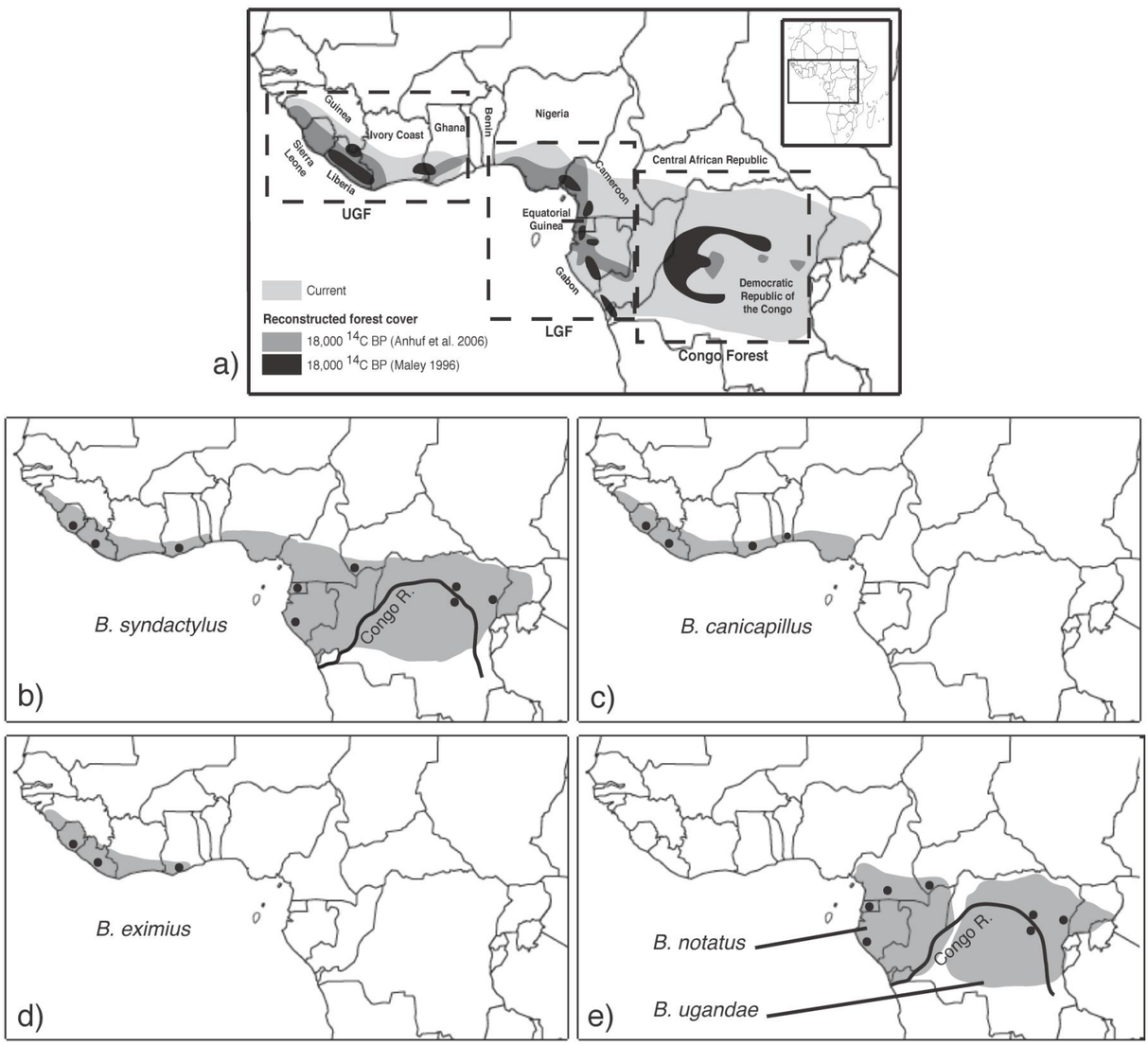


Figure 5. a) Approximate current lowland forest cover as well as purported refuges from Maley 1996 and Anhuf *et al.* 2006. Range maps for currently recognized species within *Bleda* as well as general sampling points: b) *B. syndactylus* c) *B. canicapillus* d) *B. eximius* e) *B. notatus* and *B. ugandae.* (UGF = Upper Guinean Forest; LGF = Lower Guinean Forest)

Here, we further explore the systematics and biogeography of *Bleda*. To accomplish this, we bring to bear a larger dataset, both in terms of individuals, localities sampled, and molecular markers utilized (two mtDNA and three nuclear loci). The goals of the study are three-fold. First, we investigate phylogenetic relationships within the genus *Bleda*. Second, we investigate the morphologically monotypic *B. syndactylus, B. canicapillus* and *B. eximius* for patterns of genetic diversification across all major lowland forest blocks. Finally, we seek to explore the biogeographic signals found within *Bleda* in relation to the patterns and timing of diversification events within lowland forests.

III.2 Methods

III.2.1 Taxon Sampling and Molecular Data

179 tissue samples of *Bleda* were gathered from museum collections (*B. syndactylus* = 63, *B. notatus* = 33, *B. eximius* = 21, *B. ugandae* = 18, *B. canicapillus* = 44), with the widest possible coverage across the range of each species. In addition, five taxa were chosen from within Pycnonotidae, to which *Bleda* belongs, as outgroups. *Criniger calurus, Ixonotus guttatus, Phyllastrephus albigularis, Pycnonotus barbatus* and *Andropadus latrirostris* were all found to be closely related to *Bleda* in a recent phylogeny of the Pycnonotidae (Johansson *et al.* 2007). Whole genomic DNA was extracted from fresh tissue using proteinase K digestion according to the manufacturer's instructions (DNeasy and Qiagen). Polymerase chain reaction (PCR) was utilized to amplify two mitochondrial (mtDNA) loci: NADH-dehydrogenase-2 (ND2) and

cytochrome oxidase b (CYTB) and three nuclear loci: Transforming growth factor beta (TGF β2), Myoglobin intron-2 (MYO), Aconitase 1 intron-10 (ACO1). We used standard published primers and protocols for each gene. Single-pass Sanger sequencing, using the same primer sets as utilized for PCR, was performed using ABI Big Dye Terminator v3.1 at the Beckman-Coulter Genomics facility (Danvers, MA). Both mitochondrial loci were aligned by eye and verified to be protein coding using Sequencer 4.9 (Gene Codes Corporation, Ann Arbor, MI). Nuclear loci were aligned using MUSCLE in the Genious 8.1 platform (Biomatters Ltd.).

III.2.2 Phylogenetic Analysis and Divergence Dating

Phylogenies were derived from two concatenated datasets: 1) a mitochondrial-only dataset with all individuals (n=179) and, 2) a subset of individuals (n=71) for which we sequenced five loci. We only sequenced nuclear loci for a subset of individuals since the primary concern of this investigation is to explore recent diversification patterns, for which nuclear loci are unsuited. PartitionFinder (Lanfear *et al.* 2014) was utilized to find best fit models of evolution and the most appropriate partitioning schemes for both datasets. Bayesian inference was performed using MrBayes 3.2.5 (Huelsenbeck and Ronquist 2001) using two runs of four Markov chain Monte Carlo (MCMC) chains of 10 million generations, sampled every 2,000 generations. Tracer v1.6 (Rambaut *et al.* 2014) was used to visualize post-run statistics and determine if stationarity was reached, as well as estimating an appropriate burn-in.

Species tree analysis and divergence dating estimates for a further reduced five gene dataset (n=27) were run in BEAST v2.3 (Drummond *et al.* 2012), using the standard BEAST template for divergence estimates and the *BEAST template for species tree analysis. A relaxed, lognormal clock was utilized with lineage substitution rates (per lineage/million years) for three of the five genes gathered from Lerner *et al.* (2011) (ND2=0.029, CYTB=0.014, TGF β2=0.0017). A substitution rate for ACO1 (0.0032) was taken from a recent divergence estimate for the genus *Prunella* (Drovetski *et al.* 2013). We utilized a substitution rate of 0.002 for MYO as previously employed by Voelker *et al.* (2015). Standard deviations (SD) for both mitochondrial genes and TGF β2 (CYTB=0.001; ND2=0.0025; TGF β2=0.0020) followed Lerner *et al.* (2011) while we used an SD of 0.0049 for ACO1 and 0.002 for MYO. Both BEAST analyses (*BEAST and standard BEAST) utilized a normal Yule process speciation prior and linear population function with constant root in two MCMC runs of 50,000,000 generations (sampled every 5,000 generations), with a 25% burn-in. Both runs were combined using LogCombiner v 2.3 (Drummond *et al.* 2012) and subsequently evaluated in Tracer v1.6 to measure the posterior effective sample size (ESS), as well as the 95% confidence interval for divergence dating. The combined tree topology was analyzed in TreeAnnotator v2.3 (Drummond *et al.* 2012).

III.2.3 Haplotype Networks

To assess phylogeographic patterns within the genus, median-joining haplotype networks based on the ND2 dataset were created using Network v4.6.1.3 (Fluxus-

Engineering) for *B. syndactylus, B. canicapillus and B. eximius* individuals, as these species showed intra-specific structure in preliminary phylogenetic analyses. The resulting network was tested using the MP post-processing option. Genetic distance measures, in the form of average uncorrected *p*-distances for the ND2 data between clades recovered from the mtDNA Bayesian analysis, were calculated using Mega6 (Tamura *et al.* 2013).

III.2.4 Historical Biogeography

Ancestral area reconstruction was performed using likelihood analysis of geographic range evolution (utilizing the dispersal-extinction cladogenesis (DEC) model) in the program LaGrange v.2.0.1 (Ree and Smith 2008). Two parameters within the DEC model are estimated, dispersal and extinction, while the cladogenesis parameter remains fixed. We also utilized the BioGeoBEARS package (Matzke a,b) in the R statistical platform. BioGeoBEARS has a second model (DEC +J) nested within the DEC model, with J being the jump dispersal parameter (allowing a daughter lineage to move to a non-adjacent area). Because the DEC + J model is nested in the DEC model, we were able to use a likelihood ratio test (LRT) to compare the outcomes of both analyses. We coded each species (including *ugandae*) as being present or absent in each of the three widely recognized lowland forest blocks: the Upper Guinean, Lower Guinean and Congo (Fig. 5).

III.3 Results

III.3.1 Phylogenetic Analyses

The mitochondrial dataset (ND2 and CYTB) contained 179 individuals and a total of 1,996 amplified base pairs (ND2=1041bp and CYTB=955), while the five gene dataset contained 71 individuals and 4,326 base pairs (mtDNA, MYO = 713bp, TGF β2 = 658bp, ACO1 = 959bp). We recovered the first strongly supported, multi-locus Bayesian trees for the genus *Bleda*. The results of both Bayesian analyses (five gene and mitochondrial-only) produced phylograms with congruent overall topology (Figs. 6 and 7). Five major clades were recovered with strong support (>98% posterior probability (PP)) in both of the MRBAYES analyses, reflecting the four currently recognized species plus *B. ugandae*. *Bleda syndactylus* is recovered as sister to all other species and *B. eximius* is sister to the remaining three species, with *B. notatus* and *B. ugandae* forming a sister species relationship. A comparison of both Bayesian trees with the nuclear-only tree showed a difference only in the position of *B. canicapillus*, albeit with slightly lower support (PP=0.92) in the nuclear-only tree. The *BEAST analysis recovered a tree which mirrored both Bayesian trees, albeit with lower posterior support at two nodes (Fig. 8).

Figure 6. Bayesian molecular phylogeny of the genus *Bleda* utilizing only the mithochondrial dataset (CYTB and ND2). Values close to the nodes represent posterior probabilities (* = posterior probability of 1.0).

notatus
ugandae
canicapillus
eximius
syndactylus
Benin
SLeone
Ghana/Liberia
Ghana
SLeone/Liberia
Ghana
DRC North
CAR and DRC South
Equatorial Guinea
Gabon
0.98
0.98

Our broader sequencing of mtDNA indicated that, with the exception of an individual from Cameroon, neither *B. notatus* nor B. *ugandae* display intraspecific structure. However, the remaining three species (*B. syndactylus*, *B. canicapillus*, and *B. eximius*) each show substantial structure (Figs. 6 and 7). *Bleda syndactylus* is divided into six highly-supported sub-clades corresponding to discrete geographical regions (Figs. 6 and 7). A clade from Sierra Leone + Liberia is sister to all other sub-clades and differs considerably from them in uncorrected *p*-distance (8.6%-10.3%) while a sub-clade of individuals from Ghana is recovered as sister to the remaining four clades and differs from them by similarly high uncorrected *p*-distances (6.6%-9.0%; Table 1). Two sub-clades, one representing individuals from Gabon and the other individuals from Equatorial Guinea, are recovered as sisters and they differ by an average uncorrected *p*-distance of 3.4% (Table 1). The last two sub-clades consist of birds from the Democratic Republic of the Congo (DRC) and Central African Republic (CAR). One DRC sub-clade is comprised of individuals collected north of the Congo River while the other is comprised of those collected south of the river (with the exception of one individual carrying a north bank haplotype) and three individuals from the CAR (a country whose borders are entirely north of the Congo River) (Fig. 5). The DRC North and DRC South + CAR clades differ by an average *p*-distance of 3.4% (Table 1). The average *p*-distances between Equatorial Guinea or Gabon versus DRC clades ranged from 5.5-7.9% (Table 1).

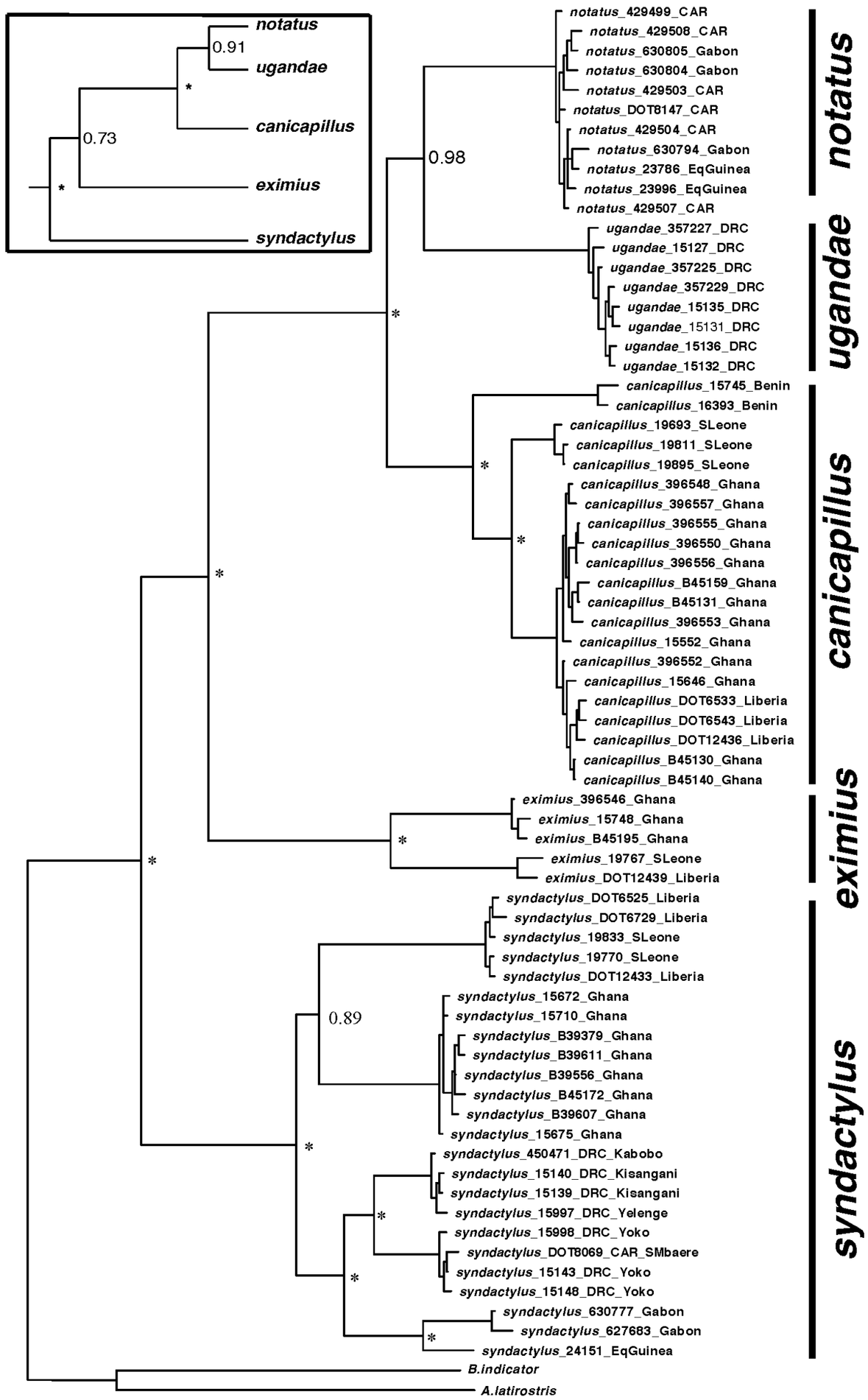


Figure 5. Bayesian molecular phylogeny of the genus *Bleda* using all five loci (two mitochondrial and three nuclear). Values close to the nodes represent posterior probabilities (* = posterior probability of 1.0). Inset: A species tree derived in *BEAST.

Sub-clades in *Bleda eximius* and *Bleda canicapillus* show similarly discrete and strongly supported geographic divisions (Figs. 6 and 7). For *B. eximius*, our results indicate the same West African break between individuals from Ghana versus those from Liberia + Sierra Leone as found in *B. syndactylus*, with a similarly high uncorrected *p*-distance of 8.5% (Table 1). The geographic structure in *B. canicapillus* is more complex than in *B. eximius*. Instead of two geographically structured sub-groups, we recover three: a Benin group, a Sierra Leone group, and a group made up of individuals from Ghana and Liberia. Average uncorrected *p*-distances for *B. canicapillus* are also lower overall than in *B. syndactylus* and *B. eximius*, with the highest genetic distance measure being between 5.9% between the Benin and Ghana sub-clades (Table 1).

Table 1. Inter and intra-specific average uncorrected pair-wise distances of ND2 for the genus *Bleda*.

	notatus	*ugandae*	*canicap*(S.L.)	*canicap* (Lib/Gha)	*canicap* (Gha)	*canicap* (Ben)	*eximius* (S.L./Lib)	*eximius* (Gha)	*syndact* (S.L./Lib)	*syndact* (Gha)	*syndact* (E.G.)	*syndact* (Gab)	*syndact* (DRC N)	*syndact* (DRC S)
notatus	-													
ugandae	0.091	-												
canicap (S.L.)	0.098	0.106	-											
canicap (Lib/Gha)	0.101	0.112	0.034	-										
canicap (Gha)	0.097	0.108	0.035	0.008	-									
canicap (Ben)	0.104	0.122	0.055	0.058	0.059	-								
eximius (S.L./Lib)	0.158	0.145	0.162	0.159	0.160	0.166	-							
eximius (Gh)	0.144	0.147	0.153	0.150	0.149	0.155	0.085	-						
syndact (S.L./Lib)	0.155	0.164	0.164	0.166	0.164	0.167	0.157	0.151	-					
syndact (Gh)	0.144	0.148	0.152	0.156	0.154	0.159	0.152	0.148	0.093	-				
syndact (E.G.)	0.146	0.152	0.158	0.162	0.159	0.157	0.143	0.147	0.096	0.085	-			
syndact (Gab)	0.145	0.151	0.150	0.152	0.151	0.155	0.145	0.150	0.092	0.090	0.034	-		
syndact (DRC N)	0.142	0.138	0.147	0.149	0.147	0.149	0.146	0.139	0.086	0.066	0.055	0.059	-	
syndact (DRC S)	0.152	0.144	0.156	0.159	0.157	0.156	0.151	0.150	0.103	0.084	0.072	0.079	0.034	-

The five-gene phylogeny reflects the same intraspecific patterns as those recovered in the larger, mtDNA-only tree: no genetic variation in *B. notatus* or *B. ugandae*, but deep and strongly supported patterns in the remaining species (Fig. 7). However, while the same six sub-clades are recovered in within *B. syndactylus*, the Ghana group is placed as sister to the Liberia + Sierra Leone clade, but with low support (0.89 PP) (Figs. 6 and 7).

III.3.2 Interspecific Divergence Dating

We note here that we are aware of the recent expansion of the Pleistocene at the expense of a contracted Pliocene. However, for historical consistency we use here the Pliocene-Pleistocene boundary of c. 1.8 Ma, as the formulation of the PFRH predates the kerfuffle (see e.g., Hilgen *et al.* 2008) regarding the assignment of the Gelasian Age to a specific Epoch.

The molecular clock analysis in BEAST yielded a tree with the same overall topology and high support as the Bayesian and *BEAST analyses. The only exception is the placement of *B. eximius* as sister to B. *syndactylus*, although this relationship was poorly supported (0.42 PP) (Fig. 8). Molecular clock estimates place two basal divergence events in the Miocene. The first is the divergence of *B. syndactylus* + *B. eximius* from all other clades (c. 7.5 Ma) and the second is the divergence of *B. syndactylus* from *B. eximius* (c. 7.2 Ma). We recover Pliocene-aged divergence events (c. 3.2 – 1.9 Ma) for several taxa within the genus, including the basal split within *B. syndactylus* of West African from central African sub-clades, the divergence of taxa in

Ghana from Liberia + S.Leone in *B. eximius*, the divergence of *B. canicapillus* and *B. notatus* + *B. ugandae*, and the divergence of *B. notatus* and *B. ugandae*.

III.3.3 Intraspecific Divergence Estimates

While all interspecific divergence events take place within the Pliocene, we do recover evidence for several intraspecific divergences within the Pleistocene (c. 1.8 Ma - 11,700 Ka) (Fig. 8). The divergence within *B. syndactylus* of the DRC North and DRC South + CAR sub-clades is estimated to have occurred c. 1.2 Ma, while the split between the Equatorial Guinea and Gabon sub-clades is recovered at 800,000 Ka. Within *B. canicapillus*, we estimate the divergence between the Benin sub-clade and all other sub-clades to have occurred c. 1.7 Ma. The divergence event between the Sierra Leone sub-clade and the Ghana + Liberia group is estimated to have an origin of c. 800,000 Ka.

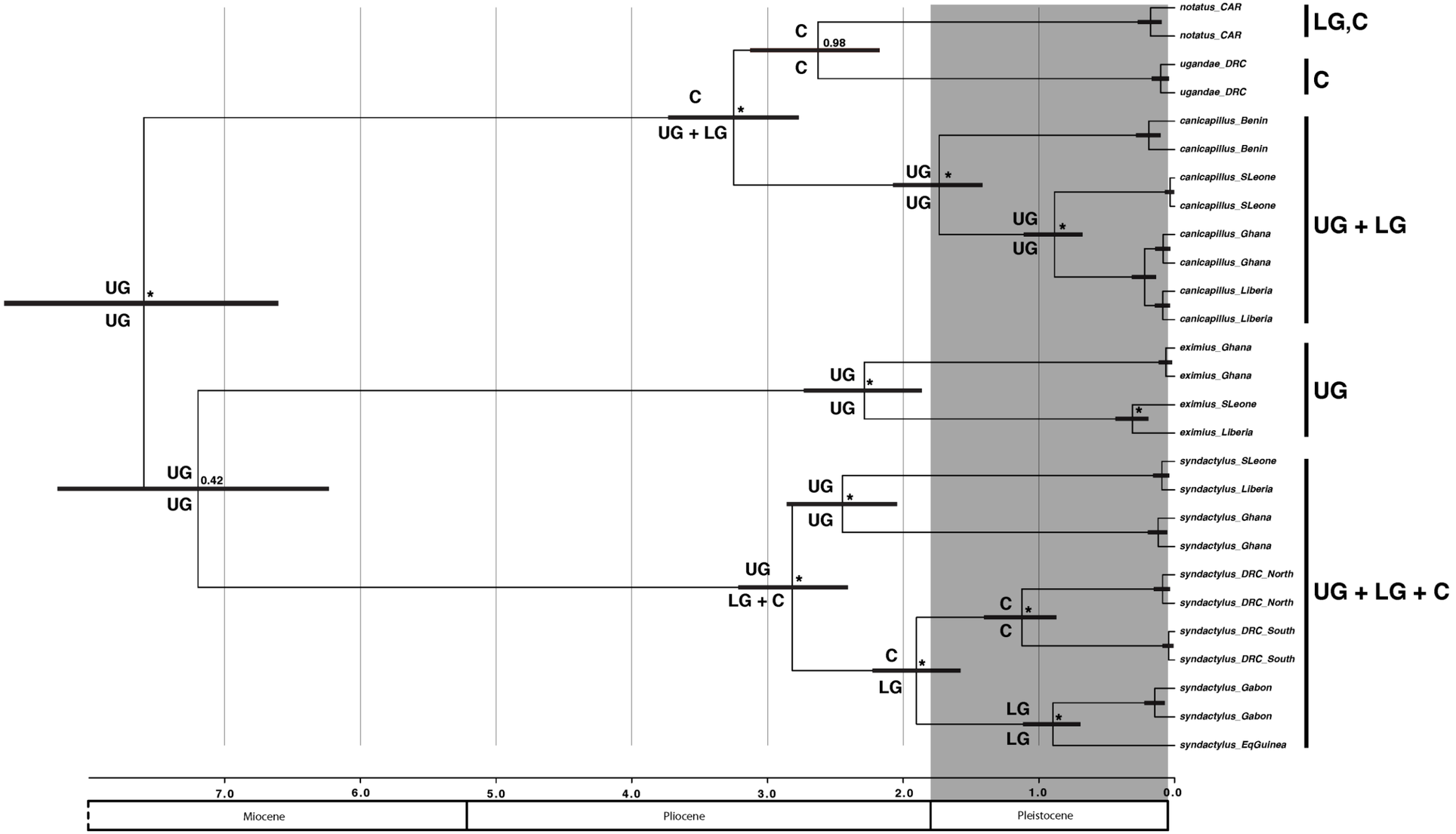


Figure 8. Molecular clock estimates of lineage divergence dates for the genus *Bleda*, based on evolutionary rates from Lerner *et al.* 2011 (CYTB, ND2, TGF β2), Drovetski *et al.* 2013 (ACO1) and Voelker *et al.* 2015 (MYO). Nodal values are posterior probabilities (* = 1.0) and nodal bars represent the 95% highest posterior density intervals. Overlayed on the figure are ancestral area estimates derived using the DEC model in LaGrange v 2.0.1. The ancestral area with the highest likelihood is represented at each node. (UG = Upper Guinean Forest; LG = Lower Guinean Forest; C = Congo Forest).

III.3.4 Ancestral Area Reconstruction

The LaGrange and BioGeoBEARS analyses provided congruent estimations for ancestral ranges for the DEC model. The DEC + J model was not shown to be significantly better (-ln 6.22; P = 0.60). Because the LaGrange and BioGeoBEARS analyses estimated congruent ancestral areas and the LaGrange visual output conformed better with our figure, we chose to only show LaGrange estimations (Fig. 8). Overall, the ancestral area reconstruction suggests a West African (Upper Guinean) origin for the basal node representing the divergence of *B. syndactylus* and *B. eximius* from the remaining species (Fig. 8). The LaGrange analysis reconstructs a pattern of western to eastern diversification within the Afrotropics, for *Bleda*. The divergence of *B. canicapillus, B. notatus* and *B. ugandae* are suggested by the analysis as having an Upper Guinean ancestral area. LaGrange reconstructs as ancestral the Upper Guinea/Lower Guinean and Congo forest blocks for the divergences between *B. canicapillus, B. notatus*, and *B. ugandae*. Our results indicate two colonizations of the Congo forest, one for *B. syndactylus* and a second for *B. notatus* + *ugandae*, as well as a potential back colonization of the Lower Guinean forest block by *B. notatus*.

III.3.5 Haplotype Networks

The ND2 haplotype networks for *B. eximius* and *B. canicapillus* show distinct haplogroups with substantial numbers of mutations between them (Fig. 9). In *B. eximius* we recover two haplogroups: one consisting of individuals from Sierra Leone + Liberia and another from Ghana; these are separated by 77 mutational steps. A Sierra Leone

haplogroup is recovered in *B. canicapillus*, but unlike the pattern demonstrated by *B. eximius*, Liberian haplotypes instead cluster with those from Ghana; the Liberia + Ghana haplogroup is separated by 28 mutational steps from the Liberia haplogroup. Also recovered within *B. canicapillus* is a Benin haplogroup, separated from the Sierra Leone haplogroup by 52 steps and from the Liberia + Ghana haplogroup by 83 steps.

The ND2 haplotype network for *B. syndactylus* produced six distinct haplogroups corresponding to the six recovered sub-clades in the Bayesian analysis: Sierra Leone + Liberia, Ghana, Equatorial Guinea, Gabon, DRC (north of the Congo River) and DRC (south of the Congo River) + CAR (Fig. 9). These haplogroups are separated by high numbers of mutational steps, as would be expected given the large *p*-distances between clades (Table 1). Overall the most western haplogroup (S.Leone/Liberia) and the DRC south + CAR haplogroup have 105 mutational steps between them.

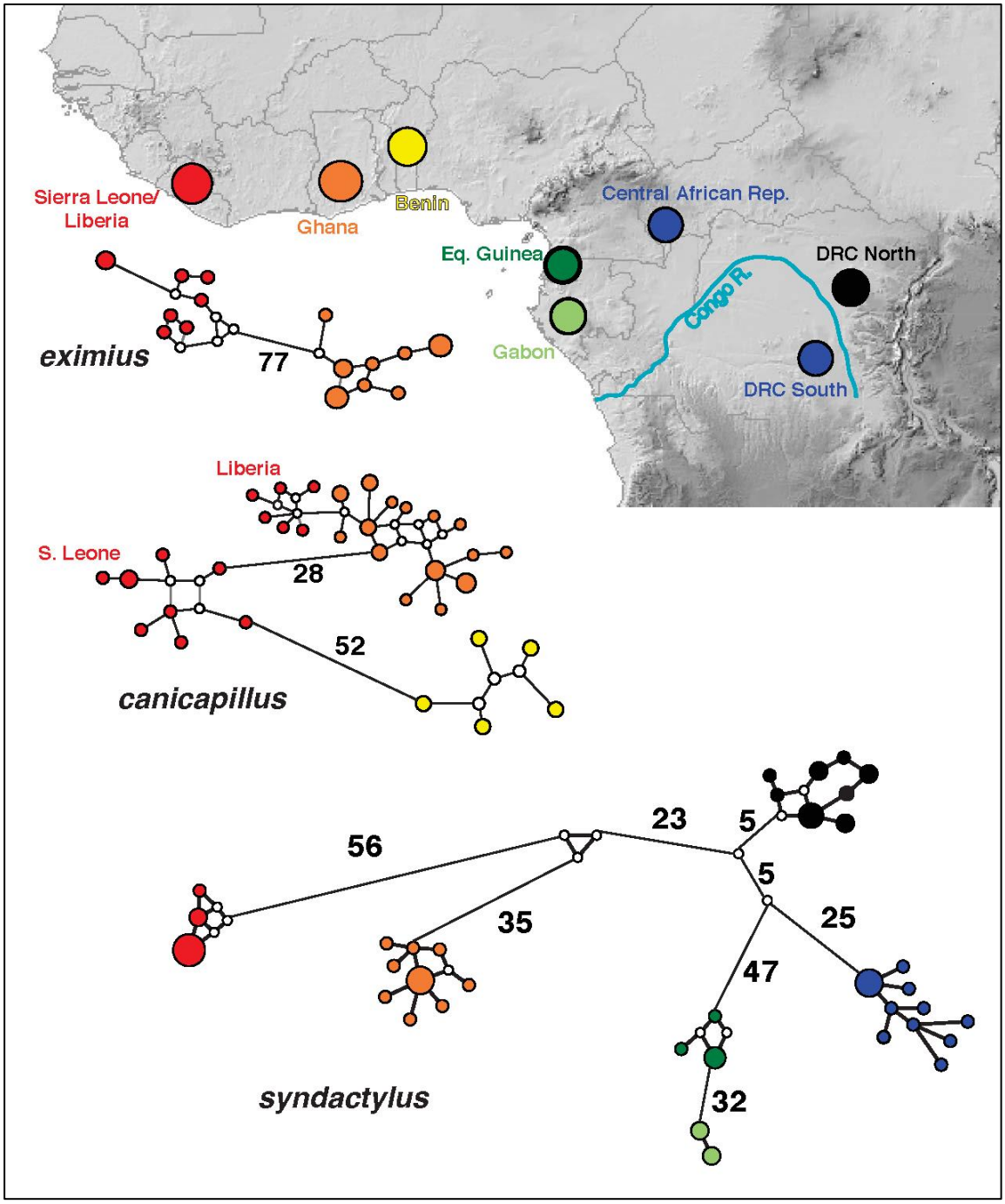


Figure 9. A median-joining network of *B. syndactylus*, *B. canicapillus*, and *B. eximius* using the ND2 dataset. Each line between haplotypes is drawn proportional to the number of mutations between haplotypes. Unfilled circles represent missing haplotypes.

III.4 Discussion

III.4.1 Systematics

We recovered the four currently recognized species of *Bleda* (*B. syndactylus*, *B. eximius, B. canicapillus, and B. notatus*) in our Bayesian analysis, while also recovering a fifth clade comprised of *Bleda ugandae* (Figure 6 and 7). Our Bayesian and *BEAST results largely mirror Beresford's (2002) results, with the exception being the recovery of *B. notatus* and *B. ugandae* as sister to one another. Beresford's results, based on a smaller dataset, recovered *B. ugandae* as sister to a *B. notatus* + *B. canicapillus* group. Beresford (2002) speculated upon the status of *B. ugandae*, stating that it may be in need of elevation to full species status based on preliminary molecular evidence. In addition to being recovered as monophyletic in all of our analyses, *Bleda ugandae* inhabits a distinct geographic range from *B. notatus*, with *B. notatus* mainly inhabiting the forest to the west of the Congo River and *B. ugandae* keeping largely to the east of the Congo River (Sinclair and Ryan 2010) (Fig. 5). Morphologically, *B. ugandae* and *B. notatus* differ most prominently in lore and eye coloration, with *B. notatus* possessing a bright yellow lore (hence the name Yellow-lored Bristle-bill) and brown eye, while *B. ugandae* (Yellow-eyed Bristle-bill) displays a distinct yellow eye and minimal yellow on the lores. Keith *et al.* (1992) noted that vocalizations between *B. notatus* and *B. ugandae* were different to the ear, but the recordings were not analyzed further. Based on our molecular data which indicate reciprocal monophyly and late Pliocene divergence from *B. notatus*, as well as its

geographic and morphological distinctiveness, it seems appropriate to recognize *Bleda ugandae* as a full species.

Beresford (2002) also suggested the possibility of *Bleda syndactylus* being comprised of multiple taxa. This suggestion was based on the deep branches which corresponded to geographic structuring. Currently, *B. syndactylus* has two recognized sub-species: *B. s. syndactylus* (Sierra Leone to western DRC) and *B. s. woosnami* (eastern DRC, S. Sudan, Uganda, W Kenya, N. Angola, and N.W. Zambia) (Keith *et al.* 1992). However, our results clearly show six distinct sub-clades characterized by deep branching patterns and high levels of genetic distance; the structure of these six sub-clades does not correspond well to the ranges circumscribed by current sub-specific limits (Figs. 6 and 9). Earlier works found initial evidence for morphological and behavioral variation within *B. syndactylus* (Louette 1991; Keith *et al.* 1992), leading to the recognition of two sub-species. These six groups certainly meet the criteria of the Phylogenetic Species Concept, but adding additional morphological and behavioral data would be preferable before naming new species or sub-species.

III.4.2 Timing of Diversification

The interspecific divergence estimates for *Bleda* (Fig. 8), given by our BEAST chronogram, are all placed within the Miocene and Pliocene epochs. This temporal pattern of speciation rejects the PFRH, a result demonstrated in several other Afro-tropical avian lineages (Bowie *et al.* 2004b; Fjeldså and Bowie 2008; Njabo *et al.*, 2008; Voelker *et al.* 2010a). Yet, the chronogram also suggests that diversification has been

continuing since the start of the Pleistocene, with five intraspecific divergences occurring in the last 1.8 my (Fig. 8). It seems that although the Pleistocene forest events may not have driven speciation (of currently accepted species), it was an important period for further diversification within several lineages of *Bleda*. This temporal result also runs counter to the claims of the "evolutionary museum" concept, which hypothesized that lowland forests are incapable of creating diversity. It is clear that much of the intraspecific variation within *Bleda* did indeed occur within lowland forests, and much of it evolutionarily recently.

III.4.3 Biogeography - West Africa

The LaGrange analysis recovered an Upper Guinean origin for *Bleda,* and the lack of any significant montane centers in that region supports the likelihood that early diversification in this group would have taken place within lowland forests. We find evidence for distinct genetic variation in West Africa between populations in Sierra Leone/Liberia and Ghana for *B. syndactylus*, *B. canicapillus*, and *B. eximius* with high genetic distances (Table 1). These two areas have long been proposed locations for Plio-Pleistocene forest refuges (Prigogine 1988; Maley 1996; Anhuf *et al.* 2006; Fig. 5). Recent studies have found similar patterns of geographic structure in Upper Guinean forests for a bird (*Hylia prasina*: Marks 2010) and several mammal species (*Lemnoscomys striatus*: Nicolas *et al.* 2008; *Crocidura olivieri*: Jacquet *et al.* 2015). It is likely that geographic structuring of genetic diversity for West African populations of *Bleda* is due, in large part, to forest fragmentation events in the Pliocene (c. 5 - 1.8 Ma)

and Pleistocene (c. 1.8 Ma - 65,000 Ka) (Figs. 5 and 8) given the lack of any apparent barriers to gene flow (e.g., large rivers or mountain ranges) between populations in Sierra Leone/Liberia and Ghana. Both of the previously mentioned mammal studies also recovered a pattern of lineage diversification associated with climate driven forest change dating to the Pleistocene epoch.

Interestingly, the pattern of haplotype relationships within *B. canicapillus* in West Africa (Sierra Leone haplogroup and a Liberia + Ghana haplogroup) does not mirror the pattern displayed by *B. syndactylus* and *B. eximius* (Sierra Leone + Liberia group and a separate Ghana group). This result is surprising given the similarity between all three species as understory specialists who almost certainly experienced the same historic fragmentation events. The *B. canicapillus* sub-clade linking individuals from Liberia with those from Ghana cannot be easily explained except with the possibility of dispersal between refuges during a fragmentation event or different sorting of ancestral haplotypes during isolation. Additionally, we recover a sub-clade of *B. canicapillus* individuals from Benin (Figs. 6 and 9), a pattern echoed closely by the recovery of a monophyletic lineage of *Stiphrornis* forest robins in a recent study (Voelker *et al.* in prep). Benin has not traditionally been associated with historic forest refuges, instead being associated with the dry, savannah conditions of the Dahomey Gap (Maley 1996). Yet the presence of unique lineages of *B. canicapillus* and *Stiphrornis* suggests the possibility of some refugial forests existing during the Pleistocene, as do recent investigations of the rodent genera *Praomys* (Bryja *et al.* 2010; Nicolas *et al.* 2011) and *Lemniscomys* (Nicolas *et al.* 2008). This suggestion is further supported by

the presence of the Lama Forest (and others) within the Dahomey Gap (White 1983) which has acted as a tropical forest refuge throughout the Holocene (c. 11,700 Ka to present) (Salzmann and Hoelzmann 2005). Additionally, the results of this study plus that on the Green Hylia (Marks 2010) are in stark contrast to several studies which have found no evidence of geographic structuring of taxa with West African distributions (Gonder *et al.* 2011; Nesi *et al.* 2013; Fuchs and Bowie 2015). Clearly there is higher than expected level of complexity in the diversification of forest mammals and birds in West Africa and recent investigations have served to highlight the limits of our knowledge of the effects of historic forest fragmentation patterns on genetic diversity in the region. More extensive sampling and further phylogeographic investigations of West African taxa will be crucial to gaining a clearer picture of the historical factors that have driven diversification in this region.

III.4.4 Biogeography - Central Africa

Several recent studies have found evidence for riverine barriers to gene flow within the Lower Guinean and Congolian Forest blocks. Voelker *et al.* (2013) found evidence for genetic differentiation in four of ten bird species with distributions on both the north and south banks of the Congo River in the eastern DRC. Two of these ten species, *B. syndactylus* and *B. ugandae*, are represented in the current study, with higher numbers of samples. We corroborate here the results found in the previous study, where *B. syndactylus* displays genetic variation north and south of the river (with 3.4% uncorrected *p*-distance) and a dating estimate for this diversification at c. 1.2 Ma. The

Congo River as a potential barrier to gene flow during the Pleistocene is not unique to birds: evidence has also been found in some rodent and bat taxa (Kennis *et al.* 2011; Bohoussou *et al.* 2015; Hassanin *et al.* 2015), either as a barrier during forest fragmentation or as a post-fragmentation barrier blocking secondary contact.

It is also clear that the Congo River is not a uniform barrier, as *B. ugandae* displays no geographic structuring across the Congo River. This is true despite having diverged (c. 2.7 Ma) well before events that led to the diversification in *B. syndactylus,* and *B. ugandae* also being an understory specialist and thus less prone to broad-scale movements than are mid- to high canopy species. In addition, we find that all three samples of *B. syndactylus* from the Central African Republic (CAR) are recovered with *B. syndactylus* samples from south of the Congo River (Figs. 5 and 9). This result is surprising given that the CAR is located well north of the Congo River and several dispersals across substantial rivers (e.g., Congo, Oubangui) are needed to explain this pattern.

The recovery of two sub-clades in *B. syndactylus* corresponding to Equatorial Guinea and Gabon populations was unsurprising given that several past investigations have placed Plio-Pleistocene refuges within both regions (Fig. 5; Maley 1996, Anhuf *et al.* 2006). In addition, and similar to the Congo River, the Ogooue River in Gabon could be acting as a barrier to gene flow between these subclades. Some species patterns have demonstrated the possibility of this river as a possible barrier to secondary contact after refugial expansion in the avian genus *Stiphrornis* (Beresford and Cracraft 1999; Schmidt *et al.* 2008) and in several mammalian taxa (Quérouil *et al.* 2003; Tefler *et al.* 2003;

Anthony *et al.* 2007; Nicolas *et al.* 2011). Greater sampling is needed in Gabon and Equatorial Guinea at finer scale to determine with certainty if this is the case in *B. syndactylus.*

III.4.5 Conclusions

The results of our investigation into the phylogeography of the genus *Bleda* coupled with similar studies of other vertebrate lowland taxa, demonstrate that Afro-tropical lowland forests are harboring a great deal more cryptic diversity than previously thought. Speciation patterns in the genus *Bleda* do not support the PFRH yet *Bleda* clearly shows the importance of Pleistocene forest fragmentation as a factor in the intra-specific diversification of this family. Certainly, the concept of the lowland forest as an "evolutionary museum" is not supported by investigations into several avian and mammalian taxa, including three of five *Bleda* lineages. These lineages explicitly show diversification and geographic structuring happening in situ in the Afro-tropical lowland forests. And while some species, such as *B. notatus* and *ugandae,* demonstrate patterns consistent with an "evolutionary museum" concept, it is clear that the MSH does not work as a sweeping explanation of genetic patterns of distribution in all African forest taxa. In addition, the idea that widespread species lacking substantial plumage variation should be discounted as uninformative (Mayr and O'Hara 1986) is certainly untrue in the case of *Bleda* as evidenced by the high levels of genetic diversification demonstrated in *B. syndactylus, B. canicapillus* and *B. eximius*. Increased collection efforts between and within forest blocks, with attention to potential genetic barriers (i.e. rivers) will be

crucial to understanding broad-scale biogeographic patterns as well as complex phylogeographic patterns within forest blocks.

III.4.6 Conservation Implications in the Afro-tropics

Understanding biogeographic patterns of African lowland forest taxa has powerful implications regarding the conservation of Afro-tropical forest biodiversity. Due to agricultural pressures, Upper Guinean forests have experienced massive destruction in the 20th century. It is estimated that ca. 80% of the original Upper Guinean forests have been either destroyed or converted in some way to agricultural use, with just 5% of the remaining forests seeing "strict" protection (Norris *et al.* 2010). This rampant forest destruction makes West African forests an urgent focal point for future conservation considerations. And though the Congo forests have been subject to a slower rate of destruction than other tropical rainforests, a serious rise in deforestation is predicted in the near future (Mosnier *et al.* 2014), making these forests a conservation priority. Unfortunately, the view of the African lowland forest as an "evolutionary museum" where little diversification has occurred has certainly contributed to conservation decision-makers treating the Congo as an area of low vulnerability (Brooks *et al.* 2006). The deep divergences and complex biogeographic patterns elucidated by analyses of the genus *Bleda*, as well as investigations of other vertebrate taxa mentioned above, have proven that the lowland forests of Africa are harboring substantial amounts of cryptic diversity, which may include new species (e.g., Voelker *et al.* 2010b). Discovering these patterns would not have been possible without the scientific collecting

efforts which have been critical to identifying regions of conservation priority in the face of habitat alteration (e.g., Bates and Voelker 2015). It is crucial that our understanding of cryptic diversification improves through further collecting efforts and biogeographic investigations, and that these investigations are considered in decisions of conservation priority. If not, we stand to lose substantial amounts of unique genetic diversity before we are even aware of its existence.

CHAPTER IV

THE IMPACT OF PLIO-PLEISTOCENE FOREST FRAGMENTATION ON AFRO-TROPICAL AVIAN DIVERSITY: THE SYSTEMATICS AND BIOGEOGRAPHY OF THE GENUS *CRINIGER* (BEARDED GREENBULS)

IV.1 Introduction

The Paleo-environmental history of the Guineo-Congolian lowland forests of Africa is characterized by retraction and expansion cycles relating to climatic oscillations of global humidity (see Plana 2004 for a review). Between the Late Miocene (~7 Ma) and Early Pliocene (~3.5 Ma), Afro-tropical forests were in an expanded state, covering substantially more land area than at present (Maley 1996). In response to a decrease in global humidity in the Early Pliocene (~3.4 Ma), Guineo-Congolian lowland forests began a major retraction phase (deMenocal 1995; Maley 1996). Since the initiation of this period of retraction, Guineo-Congolian forests have been subject to several perturbations in global humidity relating to glacial cycling, characterized by step-like shifts in the amplitude of overall aridity with peaks at 2.8 (±0.2), 1.7 (±0.1), and 1.0 (±0.2) Ma (deMenocal 2004). The consequences of this cyclical aridification on the Guineo-Congolian forests during the Plio-Pleistocene were episodes of severe fragmentation in which forests were isolated in refugial pockets. And indeed, palynological and distributional patterns of forest-dwelling taxa support the existence of multiple pockets of historical refugia throughout the Guineo-Congolian forests (Palynological: Colyn, Gautier-Hion, & Verhaven 1991; Maley 1996, 2001;

Anhuf *et al.* 2006; Distributional: Diamond & Hamilton 1980; Crowe & Crowe 1982; Mayr & O'Hara 1986; Prigogine 1988; Hamilton & Taylor 1991; Happold 1996; Levinsky *et al.* 2013). However, the size, location, and number of Plio-Pleistocene refugia remain controversial, making further biogeographic investigations of Afro-tropical forest taxa crucial to resolving this picture.

Early investigations of avian distributions across African lowland forests relied on the Pleistocene Forest Refuge Hypothesis (PFRH; Haffer 1969) as the causal agent of observed patterns (Diamond & Hamilton 1980; Crowe & Crowe 1982; Mayr & O'Hara 1986). Developed initially to explain distributional patterns in South America, the hypothesis outlines a scenario of allopatric diversification specific to the Pleistocene (defined at that time as 1.8 Ma – 11.8 Kya), in which lowland forest taxa became isolated in refugia during forest fragmentation periods, which in turn promoted allopatric speciation or in some cases, sub-specific phenotypic variation. Research assessing this hypothesis in Africa favored the investigation of 1) taxa with restricted ranges (i.e. not widespread species) and 2) taxa which display plumage variation across their range. In both instances, patterns were assumed to have derived from isolation in refugia. On the other hand, widespread species which lacked obvious phenotypic variation were viewed as "uninformative" with regards to the PFRH. Indeed, 107 taxa are specifically listed as "uninformative" in this regard by Mayr & O'Hara (1986).

An alternative refugial hypothesis, made possible with the early proliferation of molecular divergence estimates, is the Montane Speciation Hypothesis (MSH) (Fjeldså 1994; Fjeldså & Lovett 1997; Roy 1997; Roy, Sponer & Fjeldså 2001; Fjeldså *et al.*

2007; Fjeldså & Bowie 2008). These investigations revealed that many lineages of montane avifauna were relatively young (latest Miocene or younger) compared with lowland forest lineages which were dated as "ancient" (~12-20 Ma). The MSH shifted the center of diversification events away from the lowland forests and into montane regions where topographic habitat complexity and micro-climatic stability during forest fragmentation were assumed to promote speciation. In subsequent forest expansion periods, species which diverged in the Afro-montane regions dispersed into nearby lowland forests where further diversification was minimal. The MSH views the Guineo-Congolian lowland forests as "evolutionary museums", where species accumulated over time and persisted, with little change, since the Miocene.

An early consequence of the MSH and the "evolutionary museum" concept was less interest in the lowland forests role as a diversification center which can, in part, account for the relative dearth of investigations focused on endemic lowland avifauna. However, over the past decade, investigations have begun to present a pattern that counters the "evolutionary museum" concept and provides support for a more diverse picture of lowland forest diversity (*Illadopsis*: Nguembock *et al*. 2009; *Sheppardia*: Voelker, Outlaw, & Bowie 2010; *Stiphrornis*: Voelker *et al*. 2016b). For instance, the widespread, phenotypically monotypic Green Hylia (*Hylia prasina*) displays deep genetic divergences linked to highly discreet geographic structure across Afro-tropical forest blocks (Marks 2010). A recent investigation of the avian genus *Bleda*, a lowland forest endemic, found substantial geographic structuring and genetic divergences dating to the Plio-Pleistocene in three of the five species of that genus (*B. syndactylus*, *B.*

eximius, *B. canicapillus*; Huntley & Voelker 2016). Additionally, varying levels of cryptic diversification have also been demonstrated in several mammalian taxa (*Sylvisorex*: Quérouil *et al*. 2003; *Lemniscomys*: Nicolas *et al*. 2008; *Praomys*: Bryja *et al*. 2010 and Nicolas *et al*. 2011; *Crocidura*: Jacquet *et al*. 2015; *Grammomys*: Bryja *et al*. 2016; *Manis tricuspis*: Gaubert *et al*. 2016).

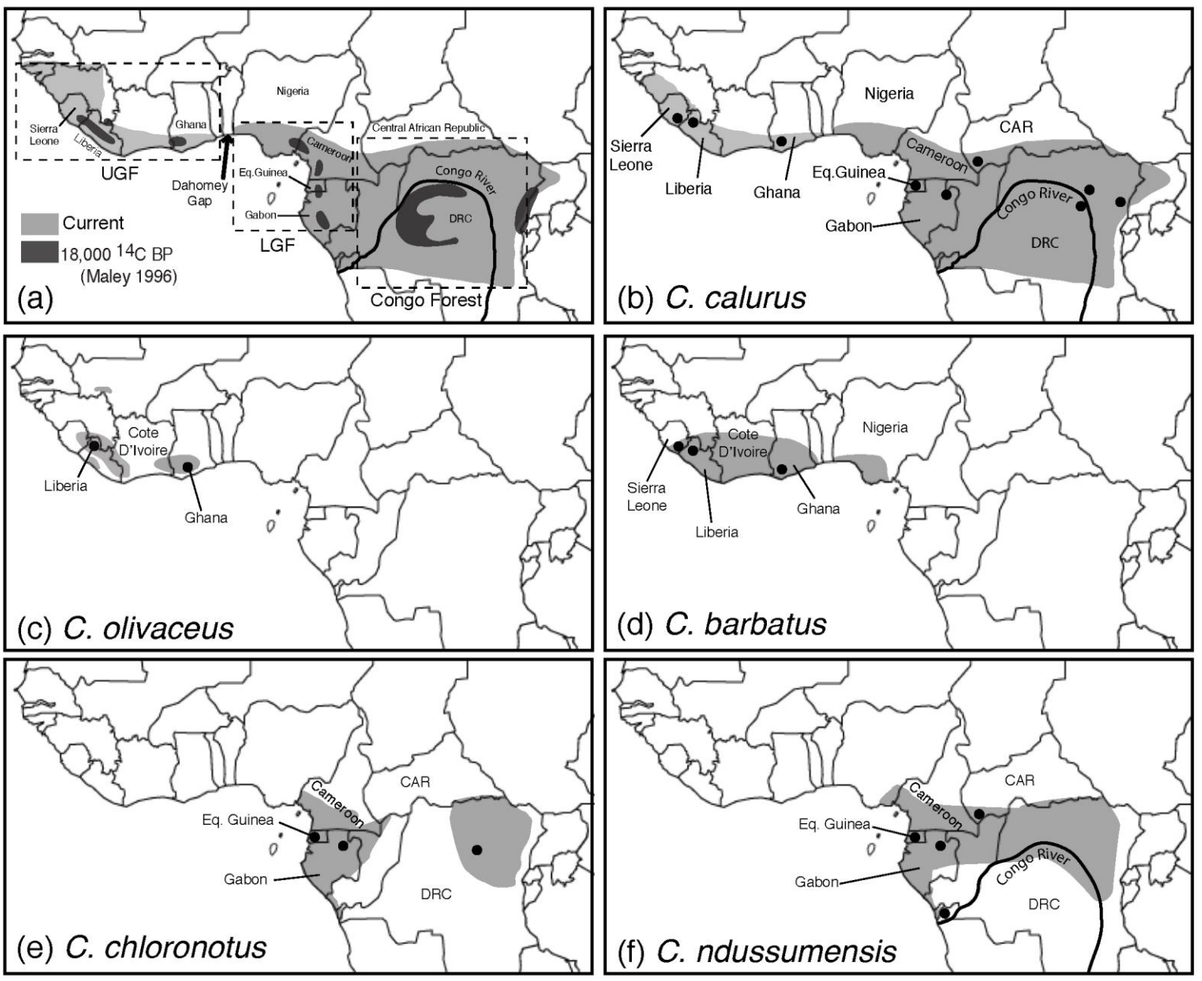


Figure 10. a) Approximate current Guineo-Congolian lowland forest cover and locations of forest refugia during the Last Glacial Maximum, as suggested by Maley 1996, as well as dashed delineations of traditional lowland forest blocks. Range maps for currently recognized species within *Criniger* along with sampling points: b) *C. calurus* c) *C. olivaceus* d) *C. barbatus* e) *C. choloronotus* and f) *C. ndussumensis.* Forest blocks are abbreviated as follows: UGF = Upper Guinean Forest block; LGF = Lower Guinean Forest block. Country abbreviations are as follows: DRC = Democratic Republic of the Congo; CAR = Central African Republic.

Alternatively, and in conjunction with traditional refugial scenarios, two additional sources of genetic diversification have been demonstrated across Guineo-Congolian forest blocks. First, early biogeographers pointed out the disjunction between West African and Central African species distributions (Diamond & Hamilton 1980; Crowe & Crowe 1982; Mayr & O'Hara 1986). It has long been hypothesized that the Dahomey Gap (Fig. 10a), a broad savannah corridor breaking the lowland forests into two blocks (Salzmann & Hoelzmann 2005), has been a major barrier to gene flow in the region. Recent investigations have recovered geographic structuring supporting an east-west vicariance scenario for several avian species (*Campethera*: Fuchs & Bowie 2015; *Dicrurus*: Fuchs, Fjeldså, & Bowie 2017). Secondly, the Riverine Barrier Hypothesis (RBH) proposes that large river systems may act as barriers to gene flow for populations across rivers (Wallace 1852). Evidence for the RBH in Africa was demonstrated by a comparative study which recovered genetic divergence patterns in four out of ten avian species distributed across the Congo River (Voelker *et al*. 2013). More broadly, investigations of several vertebrate taxa with ranges straddling other substantial river systems (e.g. the Niger, Sanaga, and Ogooué Rivers) have found evidence for varying levels of diversification supporting the RBH (rodents: Kennis *et al*. 2011; Nicolas *et al*. 2012; Bohoussou *et al*. 2015; Jacquet *et al*. 2015; bats: Hassanin *et al*. 2014; birds: Fuchs & Bowie 2015; Fuchs *et al*. 2017; Huntley & Voelker 2016).

Collectively, these recent studies cast doubt on the veracity of the "evolutionary museum" concept as a sweeping explanation for patterns of diversity in Guineo-Congolian lowland forests, and instead point to lowland forests as regions harboring

complex genetic patterns. Therefore, it is important that further investigations of African forest taxa be undertaken to determine the scope and potential role of historic mechanisms within lowland forests in creating genetic diversity. The Bearded Greenbuls (genus: *Criniger*), provide an excellent model for further investigations of lowland forest patterns. This genus formerly consisted of 10 Afro-Asian species, but recent investigations of the family Pycnonotidae found evidence for moving the Asian species into a separate genus (*Alophoixus), thus making Criniger an entirely African group (Pasquet et al. 2001; Moyle & Marks 2006). Criniger* currently consists of five understory bird species all endemic to Afro-tropical lowland forests. The Red-tailed Greenbul (C. *calurus*) is a widespread species inhabiting all regions of the Guineo-Congolian lowland forests (Fig. 10b). Two members of *Criniger* are West African endemics, the Yellow-bearded Greenbul (*C. olivaceus*) inhabiting the Upper Guinean forests (Fig. 10c), and the Western Bearded Greenbul (*C. barbatus*) which can be found in both the Upper and a relatively small portion of the Lower Guinean forests (Fig. 10d). The Eastern Bearded Greenbul (*C. chloronotus*) displays a disjunction within its range, with two main populations separately inhabiting areas east and west of Cote d'Ivoire (Fig.10e). However, it is possible that this disjunct pattern is artificial, given the general scarcity of avian records from the Cote d'Ivoire. The final species, the White-bearded Greenbul (*C. ndussumensis*), inhabits both the Lower Guinean and Congo forests, while staying mostly north of the Congo River within the latter (Fig. 10f).

To date, no investigation of the molecular phylogenetics of the genus *Criniger* has been published. While morphological and ecological evidence has traditionally

recognized at least four species, with *C. barbatus* and *C. chloronotus* as sister species (Sibley & Monroe 1990; Keith 1992; Dowsett & Forbes-Watson 1993), several hypotheses regarding the taxonomic status and relationship of *C. ndussumensis* to other *Criniger* species have been offered. *Criniger ndussumensis* has been considered to be conspecific with *C. olivaceus* (Dowsett & Forbes-Watson 1993; Dowsett-Lemaire & Dowsett 2001), a sub-species of *C. olivaceus* (Dowsett & Dowsett-Lemaire 1991), or completely invalid as a taxon (Brosset & Erard 1986). A molecular phylogenetic investigation by Beresford (2002) utilizing the mitochondrial cytochrome-*b* (CYTB) gene and a fragment of the nuclear beta-fibrinogen gene, recovered evidence confirming 1) the sister relationship between C. *barbatus* and *C. chloronotus* and 2) that *C. ndussumensis* deserves full species status. However, despite the adoption of the result of Beresford's molecular investigation by most current classifications, the study was never published. Therefore we feel it vital to re-visit earlier hypotheses within the genus *Criniger* with a larger molecular dataset than utilized by Beresford.

Here we undertake the first investigation of the systematics and biogeography of the genus *Criniger*. As understory dwelling birds with low vagility, *Criniger* species are potentially more susceptible to forest fragmentation events and as such are well positioned to retain the stamp of past refugial-driven diversification events. Furthermore, one genus member, *Criniger calurus*, is a widespread morphologically monotypic species specifically included in Mayr & O'Hara's (1986) list of 107 "uninformative" taxa. Our study goals are three-fold. First, we examine the molecular phylogenetics of the genus *Criniger*. Second, we explore the biogeographic patterns

displayed within *Criniger* in relation to broad patterns and diversification timing within lowland forests. Third, we investigate the phylogeography of each species for patterns of genetic diversification across Guineo-Congolian lowland forest blocks, with the results from *C. calurus* being a direct test of Mayr & O'Hara's (1986) assertion that widespread species lacking geographically-structured, phenotypic variation are "uninformative" for investigations of historic Afro-tropical forest diversification.

IV.2 Methods

IV.2.1 Taxon Sampling and Molecular Data

We gathered 43 tissue samples of *Criniger* from museum collections (*C. calurus* = 24, *C. ndussumensis* = 7, *C. chloronotus* = 4, *C. olivaceus* = 4, *C. barbatus* = 4). In addition, four taxa were chosen from within Pycnonotidae, to which *Criniger* belongs, as outgroups (*Andropadus latirostris, Phyllastrephus albigularis, Bleda syndactylus and Baeopogon indicator*). These species were all found to be closely related to *Criniger* in a recent phylogeny of the Pycnonotidae (Johansson *et al*. 2007). We extracted whole genomic DNA from fresh tissue using proteinase K digestion according to the manufacturer's instructions (DNeasy Blood and Tissue Kit, Qiagen, Valencia, CA). We used polymerase chain reaction (PCR) to amplify two mitochondrial (mtDNA) loci: nicotinamide adenine dinucleotide dehydrogenase subunit-2 (ND2) and cytochrome oxidase *b* (CYTB) and two nuclear loci: transforming growth factor β2 intron-5 (TGF β2), Myoglobin intron-2 (MYO). We used standard published primers and protocols for each gene. Bidirectional single-pass Sanger sequencing, using the same primer sets as

utilized for PCR, were performed using ABI Big Dye Terminator v3.1 at the Beckman-Coulter Genomics facility (Danvers, MA). Both mitochondrial loci were aligned by eye and translation was verified using Sequencer 4.9 (Gene Codes Corporation, Ann Arbor, MI). Nuclear loci were aligned using MUSCLE in the Geneious 8.1 platform (Biomatters Ltd.; http://www.geneious.com, Kearse *et al*. 2012).

IV.2.2 Phylogenetic Analysis and Divergence Dating

Phylogenies were derived from two concatenated datasets: 1) a mitochondrial-only dataset with all individuals (n=43) and, 2) a subset of individuals (n=24) for which we sequenced four loci. We used PartitionFinder (Lanfear *et al*. 2014) to determine best fit models of evolution and the most appropriate partitioning schemes for both datasets. Maximum likelihood (ML) analyses were performed using RAxML 8.0 (Stamatakis 2014) using the GTR+G model option and support was assessed using the rapid bootstrap algorithm with 1,000 replicates. Bayesian inference was performed using MrBayes 3.2.5 (Huelsenbeck & Ronquist 2001) using two runs of four Markov chain Monte Carlo (MCMC) chains of 10 million generations, sampled every 2,000 generations. Tracer v1.6 (Rambaut *et al*. 2014) was used to visualize post-run statistics and determine if stationarity was reached, and for estimating an appropriate burn-in.

Species tree analysis and divergence dating estimates for a further reduced four gene dataset (n=24) were run in BEAST v2.3 (Drummond *et al*. 2012), using the standard BEAST template for divergence estimates and the *BEAST template for species tree analysis, respectively. We note here that no fossil calibration is available for

Criniger, therefore a relaxed, lognormal clock was utilized with lineage substitution rates (per lineage/million years) for three of the four genes gathered from Lerner *et al*. (2011) (ND2=0.029, CYTB=0.014, TGF β2=0.0017) and set a substitution rate of 0.002 for MYO as previously employed by Voelker *et al*. (2016a). Standard deviations (SD) for both mitochondrial genes and TGF β2 (CYTB =0.001; ND2=0.0025; TGF β2=0.0020) followed Lerner *et al*. (2011) while we used an SD of 0.002 for MYO. Both BEAST analyses (standard BEAST and *BEAST) utilized a normal Yule process speciation prior and linear population function with constant root in two MCMC runs of 50,000,000 generations (sampled every 2,500 generations), with a 25% burn-in. Both runs were combined using LogCombiner v 2.3 (Drummond *et al*. 2012) and subsequently evaluated in Tracer v1.6 to measure the posterior effective sample size (ESS), as well as the 95% confidence interval for divergence dating. The combined tree topology was analyzed in TreeAnnotator v2.3 (Drummond *et al*. 2012).

IV.2.3 Haplotype Networks

To further assess phylogeographic patterns within the genus *Criniger*, we created median-joining haplotype networks based on the ND2 dataset using Network v4.6.1.3 (Fluxus-Engineering) for *C. calurus, C. chloronotus* and *C. barbatus* individuals, as these species showed discrete intra-specific structure in preliminary phylogenetic analyses. The resulting network was tested for unnecessary median vectors using the MP post-processing option. Genetic distances, in the form of average uncorrected *p*-

distances for the ND2 data among clades recovered from the mtDNA Bayesian analysis, were calculated using Mega6 (Tamura *et al*. 2013).

IV.2.4 Historical Biogeography

Ancestral range estimation was performed using the BioGeoBEARS package in the R statistical program (Matzke 2013a,b). We made use of BioGeoBEARS ability to run two basic models of ancestral area reconstruction, the DEC and DEC + J models. The DEC (dispersal-extinction cladogenesis) model utilizes two parameters: the dispersal rate (range expansion) and the extinction rate (range contraction), while fixing the cladogenesis model. The second model DEC + J, uses the same parameters as the DEC model while adding a third free parameter (J) corresponding to long distance dispersal. This free parameter allows one daughter lineage to move to a non-adjacent area outside of the ancestral range. We were then able to compare the two models (DEC vs. DEC + J) using a likelihood ratio test (LRT). We coded each lineage as being present or absent in the three widely recognized lowland forest blocks: the Upper Guinean, Lower Guinean and Congo forests (Fig. 10), and utilized an adjacency matrix to inform BioGeoBEARS of the geographical separation between the Upper Guinean and Congo forests.

IV.3 Results

IV.3.1 Molecular Data and Phylogenetic Analyses

The mitochondrial dataset (ND2 and CYTB) contained 42 individuals and 2,054 total amplified base pairs (bp; ND2 = 1029bp and CYTB = 1025bp), while the four gene dataset contained 24 individuals and 3,452 base pairs (mtDNA, MYO = 738bp, TGF β2 = 660bp). We recovered the first strongly supported, multi-locus ML and Bayesian trees for the genus *Criniger*. ML and Bayesian analyses of the mitochondrial-only dataset (Fig. 2) and four-gene dataset produced phylograms with congruent topologies. ML and Bayesian phylograms produced independently for each nuclear loci as well as for a combined nuclear dataset differed slightly in topology. The Bayesian MYO phylogeny recovered well-supported (posterior probability (PP) ≥ 0.97) sister relationships similar to the mtDNA and four-gene dataset, however the basal divergence results in a polytomy. The RAxML analysis produced a congruent topology for the MYO loci, albeit with moderately lower support at the break between *C. calurus* and *C. ndussumensis* + *C. olivaceus* (bootstrap value = 85). Additionally, the Bayesian TGF β2 phylogeny recovered a well-supported pattern in which C. *ndussumensis* + *C. olivaceus* is sister to the remaining species. However, while the ML analysis for TGF β2 produced identical relationships, support was generally lower. The phylogeny constructed by both the Bayesian and ML analyses is topographically congruent with the MYO tree, however support at the basal node of *C. calurus* displayed lower support (PP = 0.80; bootstrap value = 70). In both the mtDNA-only and four-gene combined phylogeny (Fig. 11) five

lineages were recovered with strong support (> 0.97 PP), reflecting the five currently recognized species of *Criniger*. We recover a phylogenetic pattern where an initial divergence separates the five clades into two major groups. The first consists of *C. calurus, C. olivaceus,* and *C. ndussumensis,* with the latter two species being sister taxa. The second major group consists of the two remaining species, *C. chloronotus* and *C. barbatus* (Fig. 11).

Both phylogenies (mtDNA-only and four-gene) reflect the same patterns of intraspecific diversification in *Criniger* (Fig. 11). The phylogeny produced by the more densely-sampled mitochondrial-only dataset shows two patterns of intraspecific diversification amongst the five recovered species: 1) limited structure with shallow divergence estimates and, 2) substantial structure with deep divergences. Pertaining to the first pattern, we find little to no structure in either *C. ndussumensis* or *C. olivaceus*, as both have shallow divergences (Fig. 11). We did recover two sub-clades for *C. ndussumensis*, one from Gabon and the other containing individuals from the Democratic Republic of Congo (DRC), Central African Republic, (CAR) and Equatorial Guinea; however, these sub-clades differ by just 0.2% uncorrected *p*-distance (Table 1) and as such have no support (Fig. 11). *Criniger olivaceus* displays limited structure between a Liberian group and two individuals from Ghana, separated by an uncorrected *p*-distance of only 0.4% (Table 1), with no support (Fig. 11).

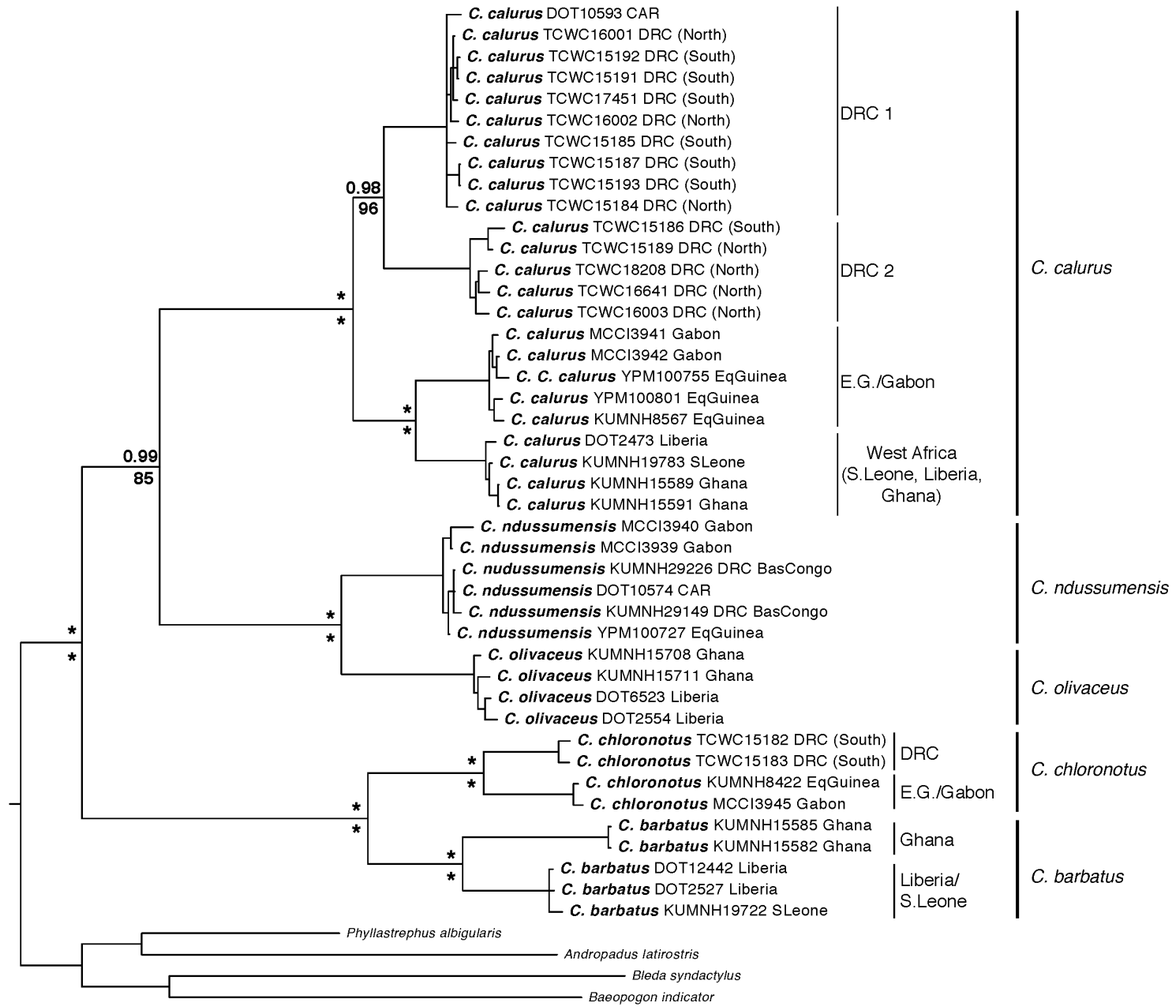


Figure 11. Bayesian molecular phylogeny of the genus *Criniger* utilizing only the mitochondrial dataset (CYTB and ND2). Values above the nodes represent posterior probabilities (* = posterior probability of 1.0) and those below represent bootstrap support values (* = 100). Country abbreviations are as follows: DRC = Democratic Republic of the Congo; CAR = Central African Republic.

Pertaining to the second pattern, the remaining three species in the mitochondrial-only phylogeny, *C. calurus*, *C. chloronotus*, and *C. barbatus*, display substantial structuring with deep divergences. The widespread *C. calurus* is divided into four highly-supported sub-clades corresponding to three discrete geographical regions (Fig. 11). We recover two sub-clades representing Congolian forest taxa (DRC1 and DRC2) as sister to two sub-clades comprising Lower Guinean forest taxa (Equatorial Guinea + Gabon) or Upper Guinean forest taxa (Sierra Leone + Liberia + Ghana; Fig. 11). These four sub-clades have substantial average uncorrected *p*-distances between them (5.8%-6.8%; Table 2). The DRC1 sub-clade is composed of six individuals collected south of the Congo River, three collected north of the river and one individual from the CAR, while the DRC2 sub-clade is composed of five individuals from the northern banks of the Congo River and only one individual from south of the river (Fig. 11). Despite the general overlap in distribution, these two DRC sub-clades are separated by an average uncorrected *p*-distance of 4.2% (Table 2). The Lower Guinean forest sub-clade (Equatorial Guinea + Gabon) is separated by an uncorrected *p*-distance of 4.7% from its Upper Guinean sister sub-clade (Table 2).

Table 2. Uncorrected pairwise distances for ND2 among and within five *Criniger* species. Country abbreviations are as follows: SLL = Sierra Leone/Liberia; EGG = Equatorial Guinea+Gabon; DRC = Democratic Republic of the Congo; CAR = Central African Republic; EG = Equatorial Guinea

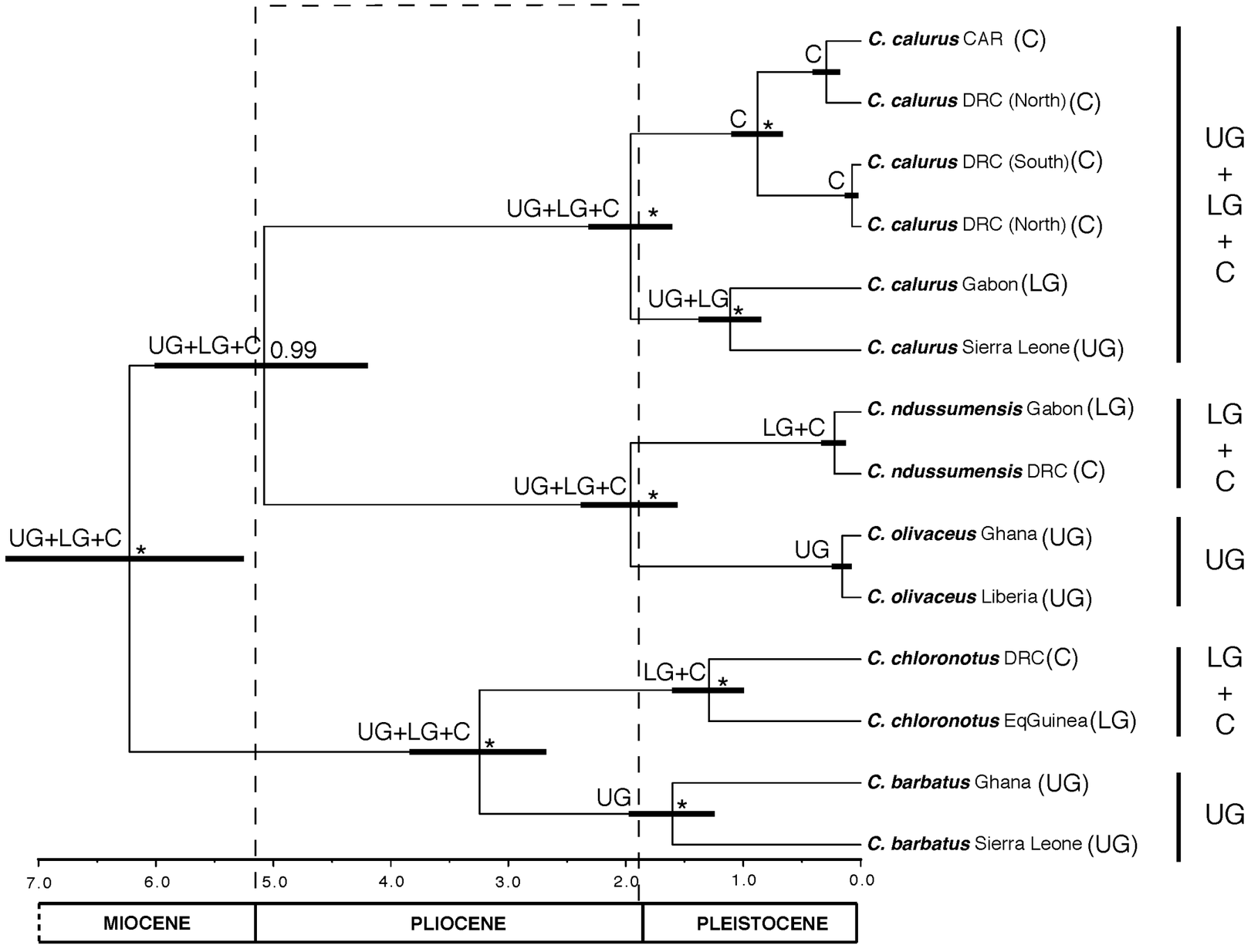
	1	2	3	4	5	6	7	8	9	10
1 *calurus* (Ghana)										
2 *calurus* (SLL)	0.001									
3 *calurus* (EGG)	0.048	0.047								
4 *calurus* (DRC1+CAR)	0.066	0.065	0.058							
5 *calurus* (DRC2)	0.069	0.068	0.062	0.042						
6 *chloronotus* (DRC)	0.136	0.137	0.131	0.137	0.138					
7 *chloronotus* (EGG)	0.142	0.141	0.138	0.142	0.141	0.052				
8 *barbatus* (Ghana)	0.139	0.140	0.128	0.141	0.144	0.092	0.103			
9 *barbatus* (SLL)	0.134	0.135	0.129	0.138	0.142	0.082	0.097	0.055		
10 *ndussumensis* (Gabon)	0.124	0.123	0.117	0.125	0.121	0.138	0.144	0.143	0.149	
11 *ndussumensis* (DRC+EG+CAR)	0.124	0.123	0.118	0.126	0.122	0.139	0.145	0.143	0.150	0.002

Sub-clades within *C. chloronotus* and *C. barbatus* show similar strongly-supported and discrete geographic structuring with substantial genetic divergence (Fig 11). A genetic break similar to *C. calurus* is recovered in *C. chloronotus*, separating Lower Guinean individuals from those in the DRC, including a similar uncorrected *p*-distance of 5.2% (Fig. 10 and Table 2). *Criniger barbatus*, an Upper Guinean forest endemic, shows a strongly supported divergence between a sub-clade of individuals from Ghana and a sub-clade from Sierra Leone/Liberia, with an average uncorrected *p*-distance of 5.5% between them (Table 2).

IV.3.2 Divergence Estimates

A recent adjustment of the Plio-Pleistocene boundary has been formally accepted, with the new beginning of the Pleistocene Epoch set at 2.58 Ma, as opposed to the historical boundary of c. 1.8 Ma. However, due to the fact that hypotheses and concepts introduced above as being relevant to the discussion of Plio-Pleistocene diversification relate to the 1.8 Ma boundary, we have chosen to use that date as the transition here, for consistency of context.

Figure 12. Species tree from *BEAST with molecular clock estimates of lineage divergence dates for the genus *Criniger*, based on evolutionary rates from Lerner *et al*. 2011 (CYTB, ND2, TGF β2) and Voelker *et al*. 2016a (MYO). Nodal values are posterior probabilities (* = 1.0) and nodal bars represent the 95% highest posterior density intervals. BioGeoBEARS ancestral range estimations are represented at each node and current recognized range is bracketed for each species. Areas are defined as follows: UG = Lower Guinean; LG = Lower Guinean; C = Congo. Country abbreviations are as follows: DRC = Democratic Republic of the Congo; CAR = Central African Republic.


C. calurus CAR (C)
C
C. calurus DRC (North) (C)
C
*
C. calurus DRC (South) (C)
C
C. calurus DRC (North) (C)
UG+LG+C
*
C. calurus Gabon (LG)
UG+LG
*
C. calurus Sierra Leone (UG)
UG+LG+C
0.99
C. ndussumensis Gabon (LG)
LG+C
C. ndussumensis DRC (C)
UG+LG+C
*
UG+LG+C
*
C. olivaceus Ghana (UG)
UG
C. olivaceus Liberia (UG)
C. chloronotus DRC (C)
LG+C
*
C. chloronotus EqGuinea (LG)
UG+LG+C
*
C. barbatus Ghana (UG)
UG
*
C. barbatus Sierra Leone (UG)
UG + LG + C
LG + C
UG
LG + C
UG
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
MIOCENE
PLIOCENE
PLEISTOCENE

The species tree analysis (*BEAST) with molecular divergence analysis recovered a strongly supported (≥ 0.99) topology congruent (Fig. 12) with both mtDNA-only and four-gene Bayesian analyses. The molecular clock analysis recovered a late Miocene (~6.2 Ma) basal divergence within *Criniger*. We recover Pliocene ages for the divergence of *C. calurus* from *C. ndussumensis* + *C. olivaceus* (~5.2 Ma) and between *C. barbatus* and *C. chloronotus* (~3.2 Ma; Fig. 12). The initial diversification of *C. calurus* lineages and the divergence of *C. ndussumensis* and *C. olivaceus* from a common ancestor are dated to the latest Pliocene (~1.9 Ma). All remaining intraspecific diversification events recovered within the genus are estimated to have occurred within the Pleistocene (Fig. 12).

IV.3.3 Haplotype Network

The ND2 haploytpe networks derived for *C. barbatus*, *C. calurus*, and *C. chloronotus* all display geographically discrete haplogroups separated by substantial numbers of mutations (Fig. 13). The ND2 network for *C. barbatus* (restricted to Upper Guinean forests) estimates two haplogroups: one consisting of individuals from Sierra Leone/Liberia, and another from Ghana, with 56 mutations separating them (Fig. 13b). The network for *C. calurus* (Fig. 13c) produced four distinct haplogroups corresponding to the sub-clades recovered in the Bayesian analysis: an Upper Guinean (Sierra Leone /Liberia + Ghana), a Lower Guinean (Equatorial Guinea + Gabon), and two Congo haplogroups, one representing a CAR haplotype plus five DRC individuals and one consisting of nine DRC individuals, with a substantial number of mutations (N = 44)

separating these two DRC groups. Overall, the western most haplogroup (Upper Guinean) is separated by 117 mutational steps from the DRC2 haplogroup (Fig. 13). Lastly, we recover two haplogroups for *C. chloronotus* (Fig. 13d). These are separated by 53 mutational steps, with one haplogroup representing Lower Guinean individuals (Equatorial Guinea + Gabon) and the other representing individuals from south of the Congo River in the DRC.

IV.3.4 Ancestral Area Estimates

The DEC model was determined to be significantly better than the DEC+J model using a likelihood ratio test ($X^2 = 3.84$; *p*-value (0.05) = 0.052)). Overall, the ancestral area estimation suggests a widespread origin (all three forest blocks) for the basal divergence of *C. chloronotus* + *C. barbatus* from the remaining species (Fig. 12). The analysis further estimates a scenario in which the ancestors of the *C. chloronotus* + *C. barbatus* clade, the *C. olivaceus* + *C. ndussumensis* clade, and *C. calurus* were distributed across all three forest blocks (UG+LG+C). Subsequently, our results indicate a diversification pattern in which widespread (UG+LG+C) ancestors diverged across the Dahomey Gap (Fig. 10), resulting in *C. barbatus* and *C. olivaceus* occupying the forests west of the gap (UG) and *C. chloronotus* and *C. ndussumensis* inhabiting the forests east of the gap (LG+C). For *C. calurus*, the analysis estimates the divergence of a widespread ancestor into a clade occupying the Upper Guinea + Lower Guinean forest blocks and one occupying the Congo Forest (Fig. 12).

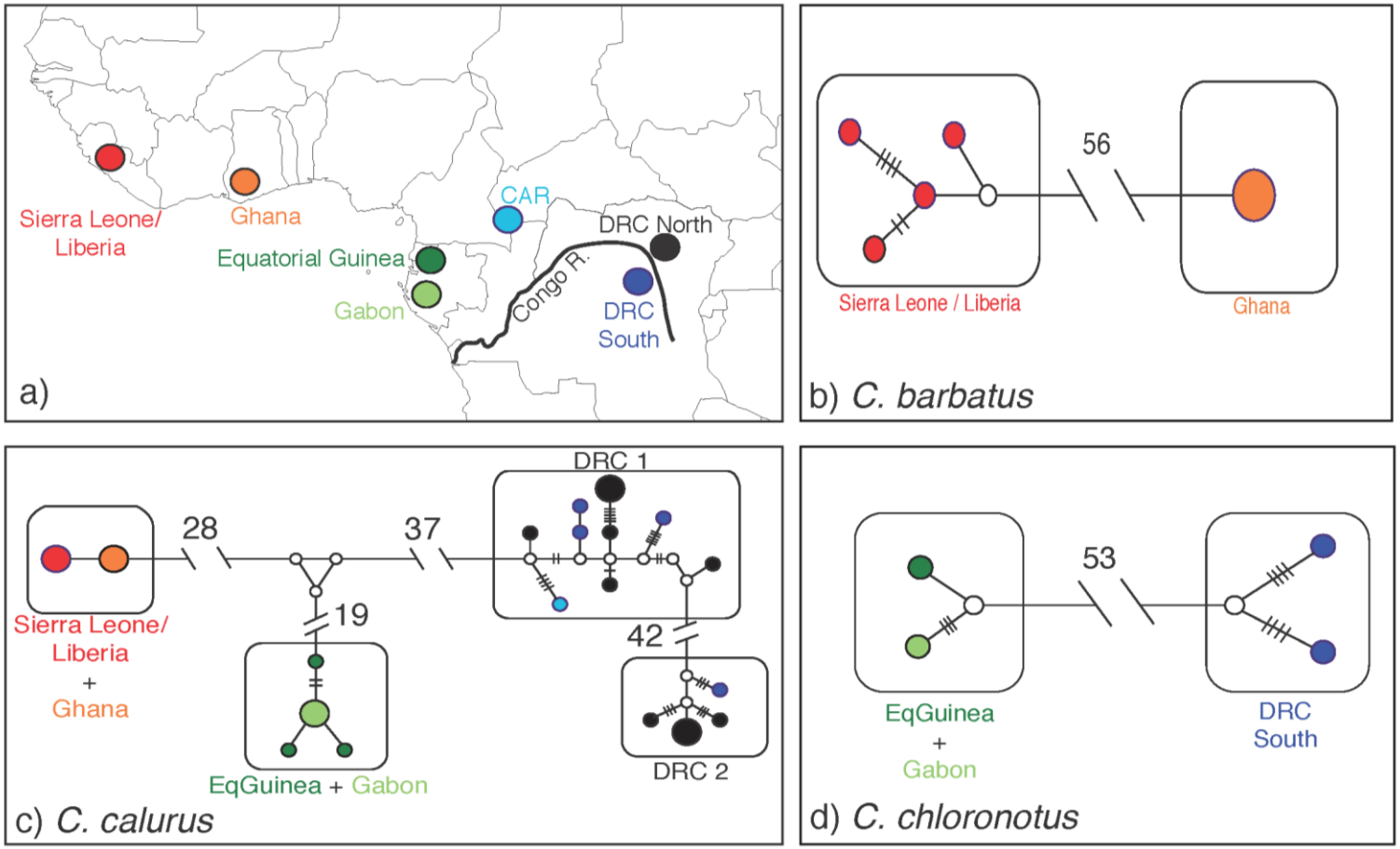


Figure 13. A median-joining network of b) *C. calurus*, c) *C. barbatus*, and d) *C. chloronotus* using the ND2 dataset. Hash marks between haplotypes represent mutational steps and lack thereof represent one mutation. Unfilled circles represent missing haplotypes. Country abbreviations are as follows: DRC = Democratic Republic of the Congo; CAR = Central African Republic.

IV.4 Discussion

IV.4.1 Systematics

Overall and in agreement with Beresford (2002), we recover all five currently recognized species of *Criniger* as strongly-supported monophyletic lineages. In congruence with current taxonomic classifications based on Beresford's study, we recover a strongly supported sister-species relationship between *C. barbatus* and *C. chloronotus* in both the mtDNA-only and four-gene phylogenies (Fig. 11 and Supplementary Figure 1). Additionally, both analyses recover a well-supported clade of *C. ndussumensis* as sister to *C. olivaceus*, with a substantial uncorrected *p*-distance between the two of ~5.5% (Table 2). Furthermore, due to the more extensive sampling regime and increased amount of molecular data than utilized in the previous study, we recover greater numbers of extensive and strongly supported sub-clades which are geographically differentiated from one another, in three *Criniger* species.

IV.4.2 Patterns and Timing of Speciation

The BioGeoBEARS analysis estimates a widespread origin (i.e. all three forest blocks) (~6.22 Ma) for the genus *Criniger* and the timing of this initial divergence is tied to early Pliocene forest retraction during the arid phase of long term climatic oscillations (Fig. 12). We then observe that the ancestors of *C. calurus* + *C. ndussumensis* + *C. olivaceus* is also estimated as widespread, having reached that range during the high humidity phase of forest expansion from the early to late Pliocene. However, the subsequent divergence event splitting *C. calurus* from *C. ndussumensis* + *C. olivaceus*

(~5.2 Ma) is difficult to explain given the state of forest expansion at this time. We suggest more intense geographic sampling of *Criniger* would be necessary to explain the causative factors of this early Pliocene-aged speciation event.

Beyond these first two divergences, the speciation patterns and timing recovered within the genus can be explained through a combination of isolation in lowland forest refugia and the breakup of the Guineo-Congolian forests into western and eastern blocks, separated by the Dahomey Gap. Indeed, several investigations have recovered similar evidence for east-west genetic diversification of avian and mammalian populations distributed across the Dahomey Gap (birds: Beresford & Cracraft 1999; Schmidt *et al*. 2008; Marks 2010; Fuchs & Bowie 2015; Huntley & Voelker 2016; mammals: Gonder *et al*. 2011; Nesi *et al*. 2013; Leaché *et al*. 2014). The patterns and timing of speciation we recovered in *Criniger* also support the involvement of the Dahomey Gap, in conjunction with more regional refugial scenarios, as a driver of two speciation events. For example, our ancestral area estimation (Fig. 12) reconstructs a widespread (UG+LG+C) distribution for the ancestors of *C. barbatus* + *C. chlorontus* and *C. olivaceus* + *C. ndussumensis*, with estimated divergence times of ~3.24 Ma and ~1.96 Ma respectively, both within the forest contraction phase of the Pliocene. These divergence dates are just prior to known aridity peaks at 2.8 and 1.7 Ma, suggesting that forest retraction can be implicated as the driver of diversification between these lineages. Specifically, our ancestral area estimations suggest speciation of these lineages as a result of isolation in forest refuges west of the Dahomey Gap (*C. barbatus* and *C. olivaceus*) and east of the Dahomey Gap (*C. chloronotus* and *C. ndussumensis*; Fig. 10).

Subsequently, the distribution of *C. chloronotus* (east of the Dahomey Gap: Fig. 10) suggests the gap remained a geographic barrier for this species during recent forest expansion phases. However, the current distribution of *C. barbatus* in both Upper and Lower Guinean forests (i.e., across the gap; Fig. 10) indicates the Dahomey Gap was not a barrier to eastward expansion for that species. Further evidence for the effects of Pleistocene-aged forest fragmentation and the Dahomey Gap as a potential barrier is demonstrated in *C. calurus*, where we recover a divergence between Upper Guinean and Lower Guinean haplogroups dated to ~1.1 Ma (Figs. 11, 12, and 13c). This date aligns with the most recent Pleistocene aridity spike defined by deMenocal (2004) at 1.0 Ma, in which forests were severely fragmented. We suggest allopatric diversification driven by a climate-induced east-west refugial scenario, subsequently reinforced by the Dahomey Gap, as the causal agent for this pattern of divergence.

IV.4.3 Intra-specific Genetic Patterns in West Africa

Two refugial areas have long been proposed to exist within the Upper Guinean forest block: one straddling the border between Sierra Leone and Liberia, and one within Ghana (Prigogine 1988; Maley 1996; Anhuf *et al*. 2006; Fig. 10). We find varying levels of evidence for these refugial areas within all three species of *Criniger* inhabiting the Upper Guinean forests. *Criniger barbatus* displays a deep divergence (uncorrected *p*-distance ~5.5%; Table 2; Fig. 13b) between individuals in Sierra Leone/Liberia and those in Ghana. This divergence is estimated to have occurred approximately 1.6 Ma (Fig. 12), a date closely corresponding to one of the Pleistocene spikes in aridity and

forest fragmentation (at 1.7 Ma; deMenocal 2004). The two other species inhabiting the Upper Guinean forests, *C. calurus* and *C. olivaceus*, also display geographic structuring between Sierra Leone/Liberia and Ghana dating to the Pleistocene (Figs. 2 and 4), albeit at substantially shallower levels (uncorrected *p*-distance of 0.1% and 0.2% respectively; Table 2) than recovered in *C. barbatus*. Given the lack of any substantial geological barrier to gene flow between these historic refugial centers (i.e. major rivers or mountain formations), it seems likely the patterns observed in these three species are a result of climate-induced forest fragmentation during the Holocene (Salzmann and Hoelzmann 2005). Notably, the recovery of geographic structuring of genetic diversity between Sierra Leone/Liberia and Ghana is not surprising given that studies of several mammalian (*Lemnoscomys striatus*: Nicolas *et al*. 2008; *Crocidura olivieri*: Jacquet *et al*. 2015) and avian species (*Hylia prasina*: Marks 2010; three species of *Bleda*: Huntley & Voelker 2016) found similar patterns.

The contrast in depth of observed intra-specific genetic divergences (i.e., shallow versus deep) recovered in the West African lineages of *Criniger* (*C. barbatus*, *C. calurus*, *C. olivaceus*) may be explained in two ways. First, the amount of time these populations remained in isolation, thereby varying the effective population size and amount of accumulated genetic mutations, was different between species. Second, isolation levels during forest fragmentation events may have varied due to differing life history strategies. *Criniger barbatus* is not observed in new growth forests and is rarely documented foraging more than 5 meters off the ground, making it a true understory specialist (Fishpool & Tobias 2005). This characteristic behavior is also shared by the

three previously mentioned species of *Bleda* in which deep genetic divergences were documented between Sierra Leone/Liberia and Ghana (Huntley and Voelker 2016). True understory specialists seem more susceptible to forest fragmentation events, as they would be less likely to cross the open spaces between fragments and also less likely to disperse through sub-optimal habitat (e.g. degraded forest and woodlands). In contrast, *C. calurus* and *C. olivaceus* have been observed regularly from 5-25 meters off the ground, and can be found in regenerating forest (Fishpool & Tobias 2005). We suggest this flexibility in habitat preference and usage may have contributed to their response to fragmentation scenarios, with both *C. calurus* and *C. olivaceus* being able to disperse between refugial centers using gallery forest, edge habitat, and sub-optimal habitats. This flexibility has been cited as a possible explanation of similar shallow geographic structuring in similarly distributed avian species (*Nectarinia olivacea*: Bowie *et al*. 2004; *Platysteira peltata*/*P. cyanea*: Njabo, Bowie, & Sorenson 2008; *Sylvietta virens*: Huntley & Voelker 2017).

IV.4.4 Intra-specific Genetic Patterns in Central Africa

Within both *C. calurus* and *C. chloronotus*, we recover deep genetic breaks (Fig. 13; Table 2) between populations in Equatorial Guinea/Gabon (EGG) and the DRC (Figs. 11 and 13) with divergences estimated to have occurred near the Pliocene-Pleistocene boundary (*C. calurus*: ~1.9 Ma) or within the Pleistocene (*C. chloronotus*: ~1.3 Ma; Fig. 12). Several investigations have shown evidence for Plio-Pleistocene refuges within Equatorial Guinea, Gabon, and the DRC (Maley 1996; Anhuf *et al*.

2006). The deep divergences recovered in these two species between EGG and the DRC would suggest isolation in refugial forest fragments during the Plio-Pleistocene, a result supported by similar patterns observed in other avian taxa (*Hylia prasina*: Marks 2010; *Bleda*: Huntley & Voelker 2016). Additionally, the lower Congo River exists as a potential barrier between populations in EGG and those in the DRC. We suggest the lower Congo River could have played a role in shaping genetic structure in the region, either as a barrier during fragmentation events or as a barrier reinforcing patterns post-fragmentation, as species ranges expanded from refugia. However, the extent of the lower Congo River's influence on the patterns observed within *C. calurus* and *C. chloronotus* is difficult to discern given the limited sampling available in the area for these species. We are unaware of studies assessing the lower Congo River as a putative barrier separating EGG and DRC populations.

In contrast to the deep genetic structure recovered in *C. calurus* and *C. chloronotus*, minimal genetic structure is recovered for *C. ndussumensis* (Fig. 11) and we estimate an origin of approximately 1.95 Ma for this species (Fig. 12). This result suggests that *C. ndussumensis* experienced similar fragmentation events as the previously mentioned species displaying high genetic differentiation levels across central African forests. For instance, a similar pattern of minimal genetic geographic structuring within the Congo Forests was recovered in both *Bleda notatus* and *B. ugandae* (Huntley & Voelker 2016). We suggest the lack of deep patterns within *C. ndussumensis* may be the result of historic isolation within only one Congo forest refuge during fragmentation, a scenario which would negate the effects of allopatric divergence

observed in other species. However, given the dearth of information regarding this species' specific habitat usage, we lack the data necessary to draw more than suggestions regarding how the life history strategies of *C. ndussumensis* may affect the patterns we recovered. Additionally, we acknowledge that the shallow patterns recovered for *C. ndussumensis* in the present study may also be a consequence of poor sampling across its range.

IV.4.5 The Upper Congo River as a Genetic Barrier

Several recent studies have recovered evidence for the upper Congo River as an historic barrier to gene flow over at least the past two million years. Voelker *et al*. (2013) found evidence for genetic differentiation across the Congo River in four out of 10 avian species sampled with distributions both north and south of the upper Congo River. A subsequent study focusing on *Bleda syndactylus*, one of the species included in Voelker *et al*. (2013), reinforced the evidence for genetic differentiation north and south of the upper Congo River through more extensive sampling (Huntley & Voelker 2016). In contrast, Voelker *et al*. (2013) found no evidence for genetic diversity structure north and south of the river in *C. calurus*, a result which this study upholds with greater sampling (Figs. 11 and 13c). However, we do recover two geographically over-lapping clades of *C. calurus* within the DRC which are substantially divergent from one another (Fig. 13c; Table 2). Prigogine (1988) and Maley (1996) both suggested the existence of one large Pleistocene forest refuge in the central DRC (along and south of the Congo River) and another in the northeastern DRC (north of the Congo River; Fig. 10a). We

propose the pattern recovered in the individuals of *C. calurus* from the DRC is due to isolation in these two refuges for a long enough period of time to allow substantial genetic differentiation between the two populations. Subsequently, as forests expanded in the more humid inter-glacial period, these populations expanded to occupy their present ranges across the upper Congo River. Further analyses of these two deeply divergent populations using other data such as morphology and song, may well support the recognition of them as species. Such analyses should obviously assess the other deeply divergent *C. calurus* lineages, as well as similar deeply divergent lineages in other *Criniger* species.

IV.4.6 Conclusion

The patterns recovered in this investigation add to the growing number of studies indicating African lowland forests harbor far more cryptic diversity than previously thought. These results offer an argument against the hypothesis that the Guineo-Congolian lowland forests are "evolutionary museums", where little *in situ* genetic diversification occurs (Fjeldså 1994; Roy 1997; Fjeldså & Lovett 1997; Roy *et al*. 2001; Fjeldså *et al*. 2005; Fjeldså *et al*. 2007; Fjeldså & Bowie 2008). Additionally, and in contrast to the assertions by Mayr & O'Hara (1986), the deeply divergent, intra-specific variation recovered in *Criniger* highlights the possible utility of widespread species, which lack plumage variation, in understanding the role of historic refugial scenarios in driving avian diversity in lowland forests. In fact, of the 107 widespread taxa deemed "uninformative" in Mayr and O'Hara's (1986) study, 10 have been investigated,

including the current study, and all were found to display geographic structuring, albeit at varying levels (*Bleda eximius, B. syndactylus*: Huntley and Voelker 2016; *Campethera nivosa*: Fuchs *et al.* 2015; *Hylia prasina*: Marks 2010; *Illadopsis rufipennis*: Nguembock *et al.* 2009; *Nectarinia olivacea*: Bowie *et al.* 2004; *Platysteira cyanea*: Njabo *et al.* 2008; *Sylvietta denti*: Huntley and Voelker unpub. data; *Stiphrornis erythrothorax*: Beresford and Cracraft 1999, Schmidt *et al.* 2008; Voelker *et al.* 2016). The results from these 10 "uninformative" taxa in conjunction with investigations of several taxa with restricted ranges indicate the substantial complexity of biogeographic patterns within Guineo-Congolian lowland forests. The current study, as well as those previously cited, highlight that no sole hypothesis can operate as a singular explanation of the substantial complexity of genetic patterns recovered throughout the African lowland forests within vertebrate species. For instance, the timing and extent of intra-specific diversification events recovered in the understory-dwelling *Criniger* lend plausibility to the Pleistocene Forest Refuge Hypothesis (Haffer 1969) and Riverine Barrier Hypothesis (Wallace 1852) as possible mechanisms working in tandem to create genetic diversity. However, these results would seem to eschew the "evolutionary museums" concept. In contrast, recent studies of several more vagile species of ubiquitous Afro-tropical forest birds have recovered minimal genetic geographic structuring across widespread species (Bowie *et al*. 2004; Fuchs & Bowie 2015; Fuchs *et al*. 2017), an outcome that lends support to the "evolutionary museum" hypothesis. The disparity in patterns between understory specialists (poor dispersers) and canopy-users/generalists (better dispersers) reveals the importance of considering varying life

history strategies on species response to historic fragmentation scenarios. Overall, the evidence recovered in *Criniger* for varying levels of diversification across the Dahomey Gap, the Congo River, and recurring climate-induced historic forest fragmentation over the last ~7 Ma indicates that African Guineo-Congolian lowland forests are dynamic zones, fully capable of creating complex and often substantial levels of genetic diversity.

CHAPTER V

AFRO-TROPICAL FORESTS ARE BOTH "CRADLES" AND "MUSEUMS" OF AVIAN DIVERSIFICATION

V.1 Introduction

Researchers have long been interested in the complex, often discordant patterns regarding the tempo and extent of species diversification in the tropical forests of the world. For decades, biogeographers and evolutionary biologists have relied on explaining the creation and maintenance of genetic diversification patterns in these forests by categorizing them either as "evolutionary cradles" or "evolutionary museums" (Stenseth 1984; Jablonski 1993; Gaston and Blackburn 1996; Chown and Gaston 2000; Wiens and Donoghue 2004; Jablonski *et al.* 2006; McKenna and Farrell 2006; Moreau *et al.* 2013). The "evolutionary cradle" hypothesis regards tropical forests as centers of diversification, often implicating broad-scale habitat alterations such as river formation, mountain building, or climate-induced habitat fragmentation cycling as driving increases in species diversity. Alternatively, the "evolutionary museum" concept proposes that tropical forests and their inhabitants have been ecologically stable or uniform for long periods of evolutionary time, resulting in often older species originations and species with widespread geographic ranges and little phenotypic diversity.

In Africa, these two competing hypotheses have been applied various times over the past three decades to complicated patterns of species richness observed within avian taxa inhabiting the Guineo-Congolian lowland tropical forest. The "evolutionary cradle"

concept was proposed for this region in the form of the Pleistocene Forest Refuge Hypothesis (PFRH). First proposed for Neotropical avian species (Haffer 1969, 1974), the PFRH was clearly applicable in efforts to explain disjunct species distributions and regions of the Guineo-Congolian forests, which like the Neotropics harbor substantial numbers of endemic bird species. This hypothesis is essentially a scenario of allopatric speciation driven by large-scale, climate-induced forest fragmentation cycling (see Fig. 14 for hypothesized Pleistocene refugial scenarios across Guineo-Congolian forest). As such, the PFRH can describe the Guineo-Congolian lowland tropical forests as centers of avian diversification during the Pleistocene Epoch (~1.8 Ma – 11,700 Ka[1]) (Diamond and Hamilton 1980; Crowe and Crowe 1982; Mayr and O'Hara 1986; Prigogine 1988). In its earliest application to the Afro-tropics, the PFRH was limited to evidence derived from plumage variation in lowland forest birds (Mayr and O'Hara 1986). Notably, in their analysis Mayr and O'Hara (1986) specifically included a list of 107 species that were widely distributed across Afro-tropical forests, but lacked regional plumage variation. As such, these species were regarded as having little utility in historical biogeographic investigations and tests of the PFRH.

[1] We are aware that the Plio-Pleistocene boundary has been pushed from 1.8 Ma to 2.58 Ma (see Hilgen *et al.* 2008). However, the "cradle" and "museum" concepts as applied to the Afro-tropics, refer to the 1.8 Ma boundary and for historical consistency in our conclusions, we use that age.

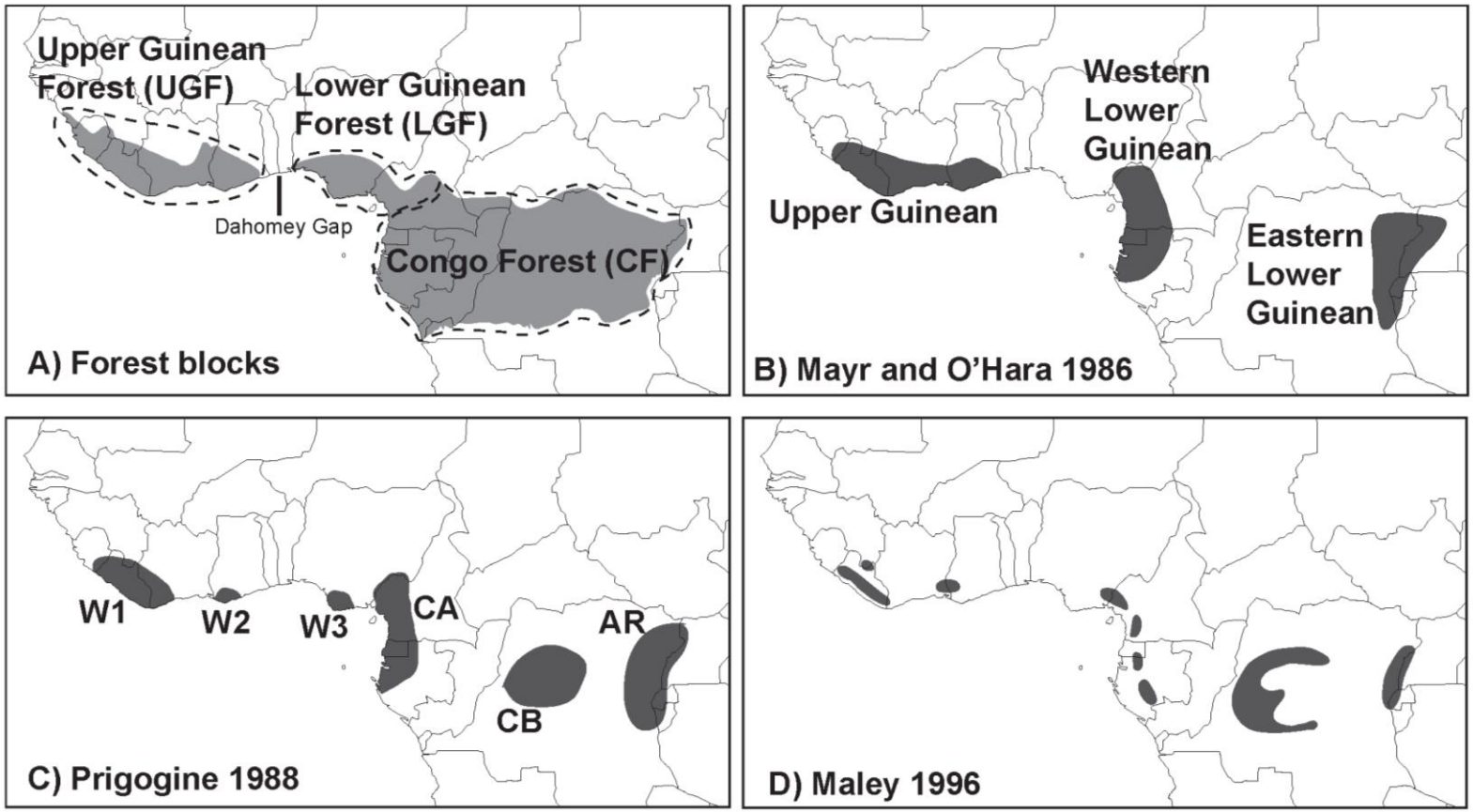


Figure 14. A) Approximate current cover of the Guineo-Congolian lowland forests and delineation of all three floristically defined blocks of the forest. Hypothesized configuration of Quaternary lowland tropical forest refugia according to: B) Mayr and O'Hara (1986), C) Prigogine 1988, D) Maley 1996.

Alternatively, a Montane Speciation Hypothesis (MSH) variant of the "evolutionary museum" concept has been proposed to explain avian species richness patterns in the Guineo-Congolian tropical forests (Fjeldså and Lovett 1997; Roy 1997; Roy *et al.* 2001; Fjeldså *et al.* 2005, 2007; Fjeldså and Bowie 2008). The inclusion of early molecular dating (relative branch lengths; Sibley and Ahlquist 1990) within a distributional analysis recovered Miocene origins (~22 Ma – 5 Ma) for a substantial majority of lowland forest species included in the analysis, contrasting much younger Plio-Pleistocene origins (≤ 5 Ma) for many montane species (Fjeldså and Lovett 1997). Based largely on these dates, the MSH proposed that Guineo-Congolian lowland forests were ecologically stable regions ("evolutionary museums") throughout the Plio-Pleistocene, as the apparent minimal genetic diversification during that time period indicated the lack of widespread mechanisms for creating significant levels of novel genetic diversity. Specifically, the MSH rejects the notion that Plio-Pleistocene refugial scenarios drove avian diversification and instead, due to substantial elevational habitat variation and stable refugia during Plio-Pleistocene aridity episodes, described the high altitude forests of Mount Cameroon and Albertine Rift Mountains as centers of diversification. In fact, the MSH explains that much of the evolutionary diversity harbored in the lowland forests are a consequence of migration from montane regions during humid phases of the global climate cycle, which drove Afro-tropical forest expansion. However, by solely utilizing inter-specific divergence comparisons, which in turn rely on morphology-based species delimitation, it is possible the MSH classification

of the lowland forests as "evolutionary museums" may be biased toward taxa judged to have completed the speciation process.

In the past decade, several investigations of Afro-tropical lowland forest birds have been published which, although not empirically testing the "cradle" versus "museum" hypotheses, found varying levels of support for both scenarios. Negligible levels of diversification have been demonstrated in several widespread avian lowland forest species which support the "evolutionary museum" concept (e.g., Bowie *et al.* 2004; Fuchs and Bowie 2015; Fuchs *et al.* 2017a; Huntley and Voelker 2017). However, several speciation events have been described which date to the Plio-Pleistocene (e.g., Schmidt *et al.* 2008; Nguembock *et al.* 2009; Voelker *et al.* 2016), and several species display deep, geographically-structured intra-specific patterns which also date to the Plio-Pleistocene (e.g., Njabo *et al.* 2008; Marks 2010; Huntley and Voelker 2016; Fuchs *et al.* 2017b). These results indicate that the Guineo-Congolian lowland tropical forests may indeed have acted both as "evolutionary museums" and "evolutionary cradles" over the past five million years. However, overall it is difficult to assess the role that the Guineo-Congolian forests have played in creating species diversity patterns (i.e., "cradle" vs. "museum") based solely on previous investigations since these studies are either restricted to a small number of species, utilize sparse geographic sampling, or concentrate on species that are ecologically and behaviorally similar.

Comparing phylogeographic patterns from 75 Afro-tropical avian lowland forest species, we investigate the extent and tempo of the creation and maintenance of genetic diversity throughout the Guineo-Congolian forests. Our goals are to assess (*i*) the extent

of intra-specific diversification patterns and tempo in lowland tropical forest birds, (*ii*) whether diversification patterns display geographic structuring, and if so whether this structuring corresponds to areas of Plio-Pleistocene refugia, and (iii) whether the timing of divergence events within species support the "evolutionary cradle" concept (recent diversification) or the "evolutionary museum" concept (ancient diversification). To accomplish these goals, we combine our own research with published data from other investigations focused on Afro-tropical forest birds. Due to large-scale and diverse taxonomic sampling, and the inclusion of species with a variety of dispersal abilities and ecological preferences, this dataset presents a unique opportunity to examine the two models of diversification that have proposed for this region across an exceptionally broad scale.

V.2 Methods

V.2.1 Molecular Data

From GenBank (www.ncbi.nlm.nih.gov/genbank), we collected 338 mitochondrial (mtDNA) sequences from 75 avian species whose primary habitat preference is defined as lowland tropical forest in the Handbook of the Birds of the World (del Hoyo 1992-2013). To minimize stochastic bias due to short sequence length, we excluded any sequences less than 500 base pairs (bp). Sequence collection yielded data from four mtDNA genes: ND2 (sequences = 151; species = 47), CYTB (sequences = 53; species = 19), ATP6 (sequences = 98; species = 3), and COI (sequences = 36; species = 7). For each of the four loci, separate alignment matrices were created in

Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI) and we used MUSCLE 3.8 (Edgar 2004) to estimate proper alignment in the two larger matrices (ND2 and CYTB). Alignments of all four loci were validated by checking for internal stop codons and confirming protein translation.

Using del Hoyo (2005) and Sinclair and Ryan (2010), species were divided into two categories: birds with low dispersal habits (i.e., understory species) or birds that are predominately dispersive (i.e., canopy and habitat generalist species). We estimated uncorrected pairwise (*p*-distances) differences between and among lineages defined below (for each locus represented) using MEGA6 (Tamura *et al.* 2013). Distance measures were estimated across three Afro-tropical forest partitions ranging from a broad to a narrow interpretation of connectivity and refugia. For the first and broadest partition, we divided sequences into two groups: those representing individuals from west of the Dahomey Gap versus those from east of the Dahomey Gap (Fig. 14A). The Dahomey Gap is a broad, arid savannah corridor located across the countries of Benin and Togo that breaks the Guineo-Congolian forests into western and eastern forests (Salzmann and Hoelzmann 2005; Fig. 14A). For the next partitioning scheme, we divided sequences into three groups corresponding to the three floristic blocks traditionally delineated within the Guineo-Congolian lowland forests: Upper Guinean Forest block (UGF), Lower Guinean Forest block (LGF), and Congo Forest block (CF; Fig. 14A).

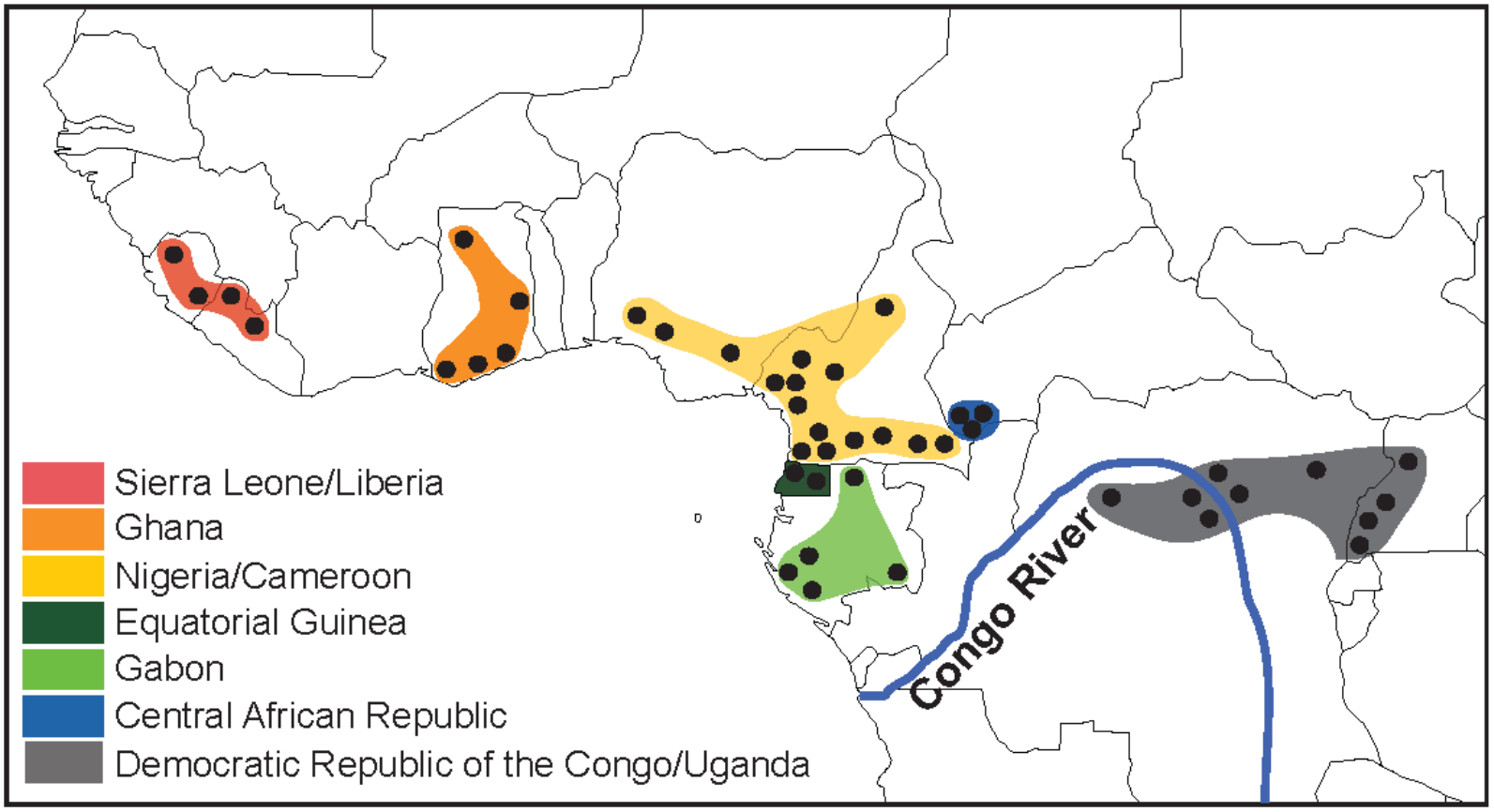


Figure 15. Delineations of intra-forest block regions from which sampling originated. Black dots represent general sampling localities.

Finally, for our narrowest partition, we grouped sequences according to the following refugial regions within each forest block: Sierra Leone/Liberia (SLL) and Ghana (both UGF); Nigeria/Cameroon (Nig/Cam, part of the LGF); Equatorial Guinea (EG), Gabon, Equatorial Guinea/Gabon (EG/G), the Central African Republic (CAR), and the Democratic Republic of the Congo/Uganda (DRC/U, all part of CF; Fig. 15).

V.2.2 Timing and Rates of Diversification

Due to the small number of species represented by the COI and ATP6 genes, we used BEAST v2.3.1 (Drummond *et al.* 2012) to estimate molecular divergence times for the ND2 and CYTB datasets only. Fossil calibrations do not generally exist for tropical forest birds, therefore we used empirical substitution rates (ND2 = 0.029; CYTB = 0.014) and standard deviations (ND2 = 0.0025; CYTB = 0.001) for each locus as derived in Lerner *et al.* (2011). These calibration values focus on songbird mutation rates; therefore, we omitted non-Passerines from both of the divergence estimate alignments, resulting in an ND2 alignment with 130 sequences from 38 species, and a CYTB alignment with 43 sequences from 14 species. BEAST analyses for both loci utilized a GTR+G evolutionary model and an uncorrelated log-normal relaxed clock with a normal Yule process speciation prior. We ran two MCMC runs of 100,000,000 generations with sampling every 1,000 steps. Each resulting logfile was examined in Tracer v1.6 (Rambaut *et al.* 2014) to ensure that effective samples sizes for all parameters were greater than 200, and to evaluate the 95% confidence intervals for divergence dating. TreeAnnotator v2.3 (Drummond *et al.* 2012) was used to find the best tree. To ensure

clock calibrations were reasonable, we compared the divergence times on the best tree to previously recovered divergence estimates from published investigations from which the sequences were derived. Times of divergence for nodes within each genus were then placed into half million year bins and a histogram of divergence times was created.

Patterns of diversification within our dataset were analyzed using BAMM (Rabosky 2014) and BAMMTools (Robosky *et al*. 2014). Our dataset contains broad taxonomic sampling but also potentially high levels of missing taxa. Due to the likelihood that diversification rate estimations may be biased in the presence of large numbers of missing taxa, we did not estimate diversification rates on the entire ND2 and CYTB phylogenies. Rather, we estimated rates for three well sampled genera: *Bleda*, *Criniger*, and *Stiphrornis*. We utilized the "setBAMMpriors" function to generate a starting prior block with BAMMTools. We specified the "speciationextinction" model, used the prior values (lambdaInit; lambdaShift; muInit) found with the "setBAMMpriors" function, and left all other parameters at their default settings. BAMM was run for 25,000,000 generations, sampled every 1,000 generations. We assessed the MCMC output for convergence by plotting the trace file and making sure all effective sample sizes were greater than 200. We then estimated the credible rate shifts that accounted for 95% of the probability of the data and from this set, and chose the shift configuration with the single best probability.

V.3 Results

V.3.1 Divergence Estimates

For our broadest Afro-tropical forest comparison, distances between lineages west and east of the Dahomey Gap (Fig. 14A), we were able to include data from 37 species (Table 1). Of these 37 species, 15 were categorized as predominately understory (low dispersers) and 22 were categorized as predominately dispersive. Within the understory species, we recovered an average mtDNA *p*-distance of 6.6% across the Dahomey Gap, with *p*-distances as low as 1.3% and as high as 13.9% (Table 3). Alternatively, we encountered lower divergence estimates across the Dahomey Gap within the dispersive species, recovering an average of 4.2% and a range of 0.1% - 11.4% (Table 3).

Table 3. Mitochondrial uncorrected pairwise distances between forests east and west of the Dahomey Gap, as well as between the Lower Guinean and Congo Forests. BOLDed species represent members of Mayr and O'Hara's (1986) list of hypothetically uninformative taxa. † = CYTB; ł = ATP6; Ł = COI; all other taxa = ND2.

Species	Forest Block	West Gap v East Gap	Upper Guinean v Lower Guinean	Lower Guinean v Congo Forest
Predominately understory				
Illadopsis cleaveri	UG, LG, CG	4.3%	0.8%	8.0%
Illadopsis rufipennis	UG, LG, CG	11.3%	11.8%	1.9%
Phyllastrephus albigularis	UG, LG, CG	1.3%	1.0%	0.9%
Andropadus latirostris	UG, LG, CG	13.9%	12.9%	14.3%
Criniger calurus	UG, LG, CG	4.5%	12.7%	12.8%
Bleda syndactylus	UG, LG, CG	8.4%	1.0%	7.7%
Alethe poliocephala†	UG, LG, CG	6.3%	7.0%	3.4%
Stiphrornis erythrothorax†	UG, LG, CG	5.0%	4.0%	6.1%
Hylia prasina	UG, LG, CG	4.2%	3.1%	4.1%
Trochocercus nitens†	UG, LG, CG	2.3%	2.2%	0.1%
Platysteira castanea	UG, LG, CG	10.5%	10.4%	1.4%
Illadopsis puveli	UG, LG	3.0%	3.0%	-
Illadopsis fulvescens	UG, CG	14.6%	-	-
Alethe diademata†	UG, CG	7.5%	-	-
Fraseria cinerascens†	UG, CG	2.3%	-	-
Andropadus virens	LG, CG	-	-	5.5%
Bleda notatus	LG, CG	-	-	2.1%
Zoothera princei†	LG, CG	-	-	0.2%
Neocossphys poensis†	LG, CG	-	-	3.3%
Sheppardia cyornithopsis	LG, CG	-	-	12.4%
Camaroptera chloronota	LG, CG	-	-	0.7%
Elminia nigromitrata	LG, CG	-	-	6.7%
Platysteira chalybea	LG, CG	-	-	1.6%
Platysteira concreta	LG, CG	-	-	0.8%
		6.6%	**5.8%**	**4.7%**
Predominately dispersive				
Alcedo leucogaster	UG, LG, CG	3.1%	3.1%	0.1%

Table 3. Continued

Species	Forest Block	West Gap v East Gap	Upper Guinean v Lower Guinean	Lower Guinean v Congo Forest
Predominately dispersive				
Halcyon malimbica	UG, LG, CG	0.1%	0.3%	0.3%
*Campethera caroli*ǂ	UG, LG, CG	2.6%	2.7%	0.4%
Campethera nivosaǂ	UG, LG, CG	5.4%	4.0%	3.0%
Dicrurus atripennis ǂ	UG, LG, CG	2.9%	3.4%	1.2%
Terpsiphone rufiventer	UG, LG, CG	2.7%	2.7%	1.5%
Platysteira cyanea	UG, LG, CG	3.2%	1.3%	5.3%
Cyanomitra olivacea	UG, LG, CG	0.6%	0.6%	0.4%
Alcedo quadribrachys	UG, LG	5.6%	5.6%	-
Tockus camurus†	UG, LG	9.4%	-	-
Tockus fasciatus†	UG, LG	11.4%	11.4%	-
Erythrocercus mccallii	UG, LG	8.7%	8.7%	-
Spermophaga haematina	UG, LG	1.0%	1.0%	-
Accipter toussenelii Ł	UG, LG	1.0%	-	-
Halcyon badia	UG, CG	1.8%	-	-
Merops gularis	UG, CG	3.7%	-	-
Merops muelleri	UG, CG	6.2%	-	-
Trachyphonus purpuratus†	UG, CG	8.7%	-	-
Psalidoprocne nitens†	UG, CG	4.3%	-	-
Sylvietta denti	UG, CG	4.2%	-	-
Sylvietta virens	UG, CG	1.3%	-	-
Muscicapa olivascens†	UG, CG	4.1%	-	-
Ispidina lecontei	LG, CG	-	-	0.3%
Sasia africana	LG, CG	-	-	0.1%
Indicator maculatus	LG, CG	-	-	0.0%
Stizorhina fraseri	LG, CG	-	-	0.0%
Muscicapa sethsmithi†	LG, CG	-	-	5.7%
Terpsiphone rufocinerea	LG, CG	-	-	0.9%
Terpsiphone batesi	LG, CG	-	-	0.9%
*Deleornis fraseri*Ł	LG, CG	-	-	3.6%
		4.2%	**3.7%**	**1.5%**

Our three forest block partition (Fig. 1B) yielded 24 species comparisons between the UGF and LGF, and 36 comparisons between the LGF and CF blocks (Table 3). We recovered relatively similar *p*-distance estimates as the Dahomey Gap comparison when we compared the UGF and LGF blocks, with 12 understory species averaging 5.8% (range: 0.8% - 12.9%) and 12 dispersive species averaging 3.7% (range: 0.3% - 11.4%). Understory species compared between the LGF and CF averaged 4.7% (range: 0.1% - 14.3%) versus a lower average genetic distance (1.5%; range: 0.0% - 5.7%) within dispersive species from the same regions (Table 3).

For our narrowest partitioning of forests (sub-blocks of UGF, LGF and CF; Fig. 14), we obtained mtDNA pairwise distance comparisons across a total of 23 understory species and 21 dispersive species (Table 4). Between Sierra Leone/Liberia and Ghana (both UGF), we calculated an average *p*-distance of 4.2% for understory species and 1.6% for dispersive species. Additionally, we observed sequence divergence estimates as high as 9.3% for understory species between these two UGF regions (Table 4). In the LGF, we observed a sequence divergence average of 6.3% in understory birds versus 1.1% in dispersive species between geographically adjacent regions around the Gulf of Guinea (Nigeria/Cameroon versus Equatorial Guinea/Gabon; Table 4). Within the Congo Forest block, our comparison between Equatorial Guinea/Gabon and the Democratic Republic of the Congo/Uganda yielded an average distance of 6.0% for understory taxa and 2.7% for dispersive taxa (Table 4). We observed a sequence divergence average of less than 2% for both species groups between Equatorial Guinea and Gabon (1.9%; 0.6%); the two most geographically close areas in our study.

Table 4. Mitochondrial uncorrected pairwise distances between regions within the Guineo-Congolian forest blocks. BOLDed species represent members of Mayr and O'Hara's (1986) list of hypothetically uninformative taxa. † = CYTB; ł = ATP6; Ł = COI; all other taxa = ND2. Abbreviations are as follows: SLL = Sierra Leone/Liberia; Nig/Cam = Nigeria/Cameroon; CAR = Central African Republic; EG/G = Equatorial Guinea/Gabon; EG = Equatorial Guinea; DRC/U = Democratic Republic of the Congo/Uganda.

Species	SLL v Ghana	Nig/Cam v CAR	Nig/Cam v EG/G	EG/G v CAR	EG/G v DRC/U	EG v Gabon	DRC/U vs CAR
Primarily Understory							
Illadopsis cleaveri	1.8%	5.9%	8.0%	-		-	-
Illadopsis fulvescens	-	-	-	-		-	10.4%
Illadopsis rufipennis	6.8%	2.2%	-	1.7%		-	-
Criniger barbatus	5.7%	-	-	-	-	-	-
Criniger calurus	0.1%	13.0%	12.2%	6.5%	6.3%	0.0%	2.3%
Criniger chloronotus	-	-	-	-	5.6%	0.6%	
Criniger ndussumensis	-	-	-	0.2%		0.4%	
Criniger olivaceus	0.6%	-	-	-	-	-	-
Bleda canicapillus	3.5%	-	-	-	-	-	-
Bleda eximius	8.5%	-	-	-	-	-	-
Bleda notatus	-	2.2%	2.1%	0.4%	-	0.4%	-
Bleda syndactylus	9.3%	10.4%	8.7%	7.2%	6.5%	3.4%	1.9%
Zoothera princei†	-	0.2%	-	-	-	-	-
Neocossphys poensis†	-	3.3%	-	-	-	-	-
Neocossyphus rufus	-	-	-	-	14.7%	-	-

Table 4. Continued

Species	SLL v Ghana	Nig/Cam v CAR	Nig/Cam v EG/G	EG/G v CAR	EG/G v DRC/U	EG v Gabon	DRC/U vs CAR
Primarily Understory							
Sheppardia cyornithopsis	-	11.8%	-	-		-	3.5%
Alethe castanea†	-	-	-	0.6%	1.5%	-	1.5%
Alethe poliocephala†	1.9%	3.0%	3.0%	0.3%	3.2%	-	3.2%
Stiphrornis erythrothorax†	3.5%	7.0%	3.9%	6.8%	6.6%	6.8%	2.3%
Hylia prasina	4.5%	2.6%	-	1.8%	4.8%	-	4.7%
Bathmocercus rufus	-	-	-	-	4.5%	-	-
Trochocercus nitens†	-	0.1%	-	-		-	-
Platysteira concreta	-	0.6%	-	0.6%	-	-	-
	4.2%	**4.8%**	**6.3%**	**2.6%**	**6.0%**	**1.9%**	**3.7%**
Primarily Dispersive							
Accipiter castanilius‡	-	-	-	-	6.5%	-	-
Alcedo leucogaster	-	0.1%	-	-	-	-	-
Halcyon malimbica	-	0.3%	-	-	-	-	-
Ispidina lecontei	-	0.1%	-	-	-	-	0.4%
Bycanistes cylindricus†	4.2%	-	-	-	-	-	-
Tricholaema hirsuta†	-	-	1.3%	0.3%	-	-	-
Campethera caroli‡	-	0.3%	0.5%	0.5%			
Campethera nivosa‡	0.1%	-	-	0.7%	0.8%	0.5%	0.2%

Table 4. Continued

Species	SLL v Ghana	Nig/Cam v CAR	Nig/Cam v EG/G	EG/G v CAR	EG/G v DRC/U	EG v Gabon	DRC/U vs CAR
Primarily Dispersive							
Sasia africana	-	-	0.1%	-	-	-	-
Hirundo nigrita†	-	-	-	-	-	0.3%	-
Dicrurus atripennis ł	-	-	1.2%	-	-	-	-
Stizorhina fraseri ł	-	0.0%	-	-	-	-	-
Sylvietta virens	-	-		0.7%	0.9%	1.3%	1.4%
Camaroptera chloronota	-		0.7%	-	-	-	
Fraseria ocreata†	-	-	-	-	-	-	4.3%
Muscicapa cassini†	-	-	-	-	-	-	11.4%
Terpsiphone rufiventer	0.6%	-	1.3%	-	-	-	-
Platysteira tonsa	-	-		-	-	0.4%	-
Lamprotornis splendidus	-	-	-	-	-	-	0.8%
Cyanomitra olivacea	-	-	0.4%	-	-	-	-
*Deleornis fraseri*Ł	-	-	3.6%	-	-	-	-
	1.6%	**0.2%**	**1.1%**	**0.6%**	**2.7%**	**0.6%**	**3.1%**

Also within the Congo Forest block, a comparison between the Democratic Republic of the Congo/Uganda and the Central African Republic produced similar average *p*-distances between understory and dispersive species, 3.7% and 3.1% respectively. However, for dispersive species this average is inflated by one species (*Muscicapa cassini*: 11.4%) and when it is removed the average falls to 1.4% (Table 4).

V.3.2 Timing and Rates of Diversifications

The BEAST analysis recovered strongly supported topologies and divergence times congruent with estimates previously established for several genera. We found low posterior probabilities (PP ≤ 99) at several nodes representing older (> 5 Ma) inter-generic lineages in which numerous species are known to be missing. Overall, divergence dates were taken from a combined 150 intra-generic nodes across the ND2 and CYTB topologies. All but eight divergences were equal to or younger than 5 Ma (Fig. 16A). Between 5 Ma – 2 Ma we observe a relatively low number of divergences, with no bin containing more than 10 divergences. However, from 2 Ma to present, we observe a general rise in diversification events, with spikes of 19 nodes between 1 Ma and 0.5 Ma and 59 nodes from 500,000 Ka to present (Fig. 16A).

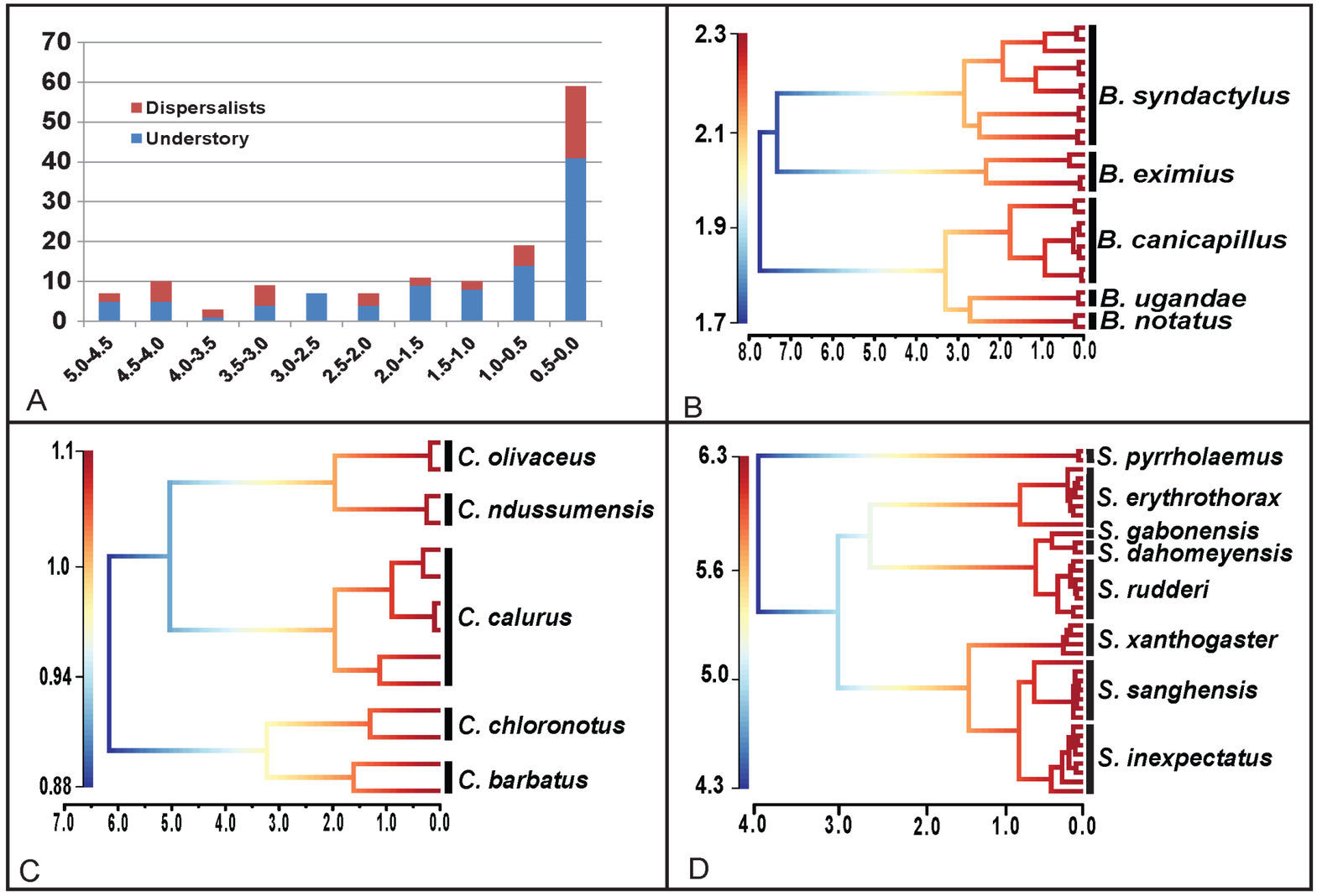


Figure 16. A) Histogram of intra-generic molecular divergence estimates younger than 5 Ma from sequences in both the ND2 and CYTB alignments (Passerine-only). Each bar represents the number of divergences occurring in that 0.5-million-year bin. B-D) Trees for *Bleda*, *Criniger*, and *Stiphrornis* showing the best single shift configuration from the diversification analysis in BAMM.

The results of our BAMM analysis on the diversification rates for three well sampled understory genera (*Bleda*, *Criniger*, and *Stiphrornis*) yielded a convergent output with effective sample sizes over 200. After filtering out models with low marginal probability, an estimation of the credible rate shifts accounting for 95% of the probability favored a single shift configuration for all three genera. BAMM estimated a single discrete core shift at the basal node for *Bleda*, *Criniger*, and *Stiphrornis*, followed by general increases in diversification rates towards the present. Specifically in the genera *Bleda* and *Stiphrornis*, we observe a nearly uniform rate of increase through time since the origin of both groups. However, in *Criniger* we estimate a steep rise in the diversification rate between the basal node and ~ 2.5 Ma, and only a small increase thereafter (Fig. 16B-D).

V.4 Discussion

Biogeographers and evolutionary biologists have long been interested in understanding the drivers of diversification patterns in Afro-tropical forests. The role of the Dahomey Gap in driving broad-scale biodiversity distributions, including the large number of endemic species inhabiting the Upper Guinean forests, has been of particular interest (Booth 1958; Endler 1982; Mayr and O'Hara 1986; Fig. 14). Investigations focusing on genetic diversification patterns found evidence for a lack of shared haplotypes across the Dahomey Gap in select avian (Beresford and Cracraft 1999; Schmidt *et al.* 2008; Marks 2010; Fuchs and Bowie 2015; Voelker *et al.* 2016; Fuchs *et al.* 2017), mammal, (Nesi *et al.* 2013; Hassanin *et al.* 2014), and reptile species (Leaché

et al. 2014). However, the extent to which the Dahomey Gap has served as a barrier to a large faunal assemblage has not been extensively examined via molecular methods, as we do here for Afro-tropical forest dwelling bird species. We found strong evidence for a genetic break in 37 avian species occupying forests to the west and to the east of the Dahomey Gap, including 24 species that inhabit the forest blocks directly adjacent to the gap (Upper Guinean and Lower Guinean). Fifteen of the total 37 species are understory dwellers, and as such are more likely to be affected by habitat discontinuities. However, we provide similar evidence for 22 dispersal-adapted species, a result that clearly indicates that this barrier has played a major role in fragmenting widespread avian populations through time (Table 3).

In addition to the Dahomey Gap, many biogeographers have hypothesized the existence of Plio-Pleistocene lowland forest refugia consisting of coastal, riverine, and sump habitats during periods of high global aridity (Fig. 14A), and discussed their potential importance for shaping avian distributional patterns and diversity (e.g., Diamond and Hamilton 1980; Crowe and Crowe 1982; Mayr and O'Hara 1986; Prigogine 1988). The spatial diversity patterns we recovered by utilizing refugial areas rather than major forest blocks for bird distributions, suggest that Plio-Pleistocene forest fragmentation played a major role in the creation of phylogeographic structure in many avian species. For instance, we observed greater than 3% sequence divergence between seven (of 11) populations of understory taxa between Sierra Leone/Liberia and Ghana in the Upper Guinean forest block (Table 4). These two regions are continuously connected by lowland forest in the Cote d'Ivoire, lack major gene flow barriers (i.e., rivers or

mountains) between them, and each is hypothesized to have been the location of Pleistocene forest refugia (Fig. 14B-D). Indeed, several other sampling locations we include are former Pleistocene refugial centers, and diversification patterns between these centers and other areas support their role as refugia between which lineages have or are diversifying (Figs. 14 and 15).

In addition, the tempo and rate of diversification we recover also supports the importance of Plio-Pleistocene climate-based landscape alterations on the creation of avian genetic diversity. Of the 150 intra-generic lineage divergences recovered in BEAST, 113 occur within the last 3 Ma (Fig. 16A), a time period for which evidence suggests Afro-tropical forests underwent major fragmentation events related to successive spikes in global aridity (deMenocal 1995, 2004; Maley 1996). Furthermore, of these 113 divergences, 78 are estimated to have occurred within the late Pleistocene to present (1.0 – 0.0 Ma; Fig. 16A). Although a number of these lineage splits may be a bi-product of available samples, many display genetic distance values that undoubtedly reflect true divergences (Tables 3 and 4). The high number of divergences for this time period is not surprising as the Late Quaternary (especially beginning approximately 800,000 Ka) was subject to high frequency fluctuations of global aridity strongly associated with glacial cycling which in turn led to severe bouts of forest fragmentation (Hewitt 1996, 2000; see Plana 2004 for review). An increasing tempo of diversification events starting at 3 Ma is further supported by our estimation of an increase in diversification rates for three densely sampled genera, since their basal divergences (Fig. 16B-D).

All previous studies of the diversification patterns of the Guineo-Congolian forests have focused on a single taxon or a small number of related taxa sharing similar dispersal abilities and ecological constraints. Our results, which utilized a large number of co-occurring avian taxa with different dispersal abilities, revealed an extraordinary complexity of genetic diversification patterns across the Guineo-Congolian lowland tropical forests over the last 3 Ma. We find that dispersal ability greatly affects a bird species' response to historic vicariance scenarios. Our distance comparison of the intra-block partition (Table 4) shows that understory species display overall deeper levels of genetic lineage divergences (1.9% - 6.3%) than more dispersive species (0.2% - 3.1%), with several dispersalist taxa displaying null levels of diversification, across broad areas. This is consistent with evidence that understory species are generally less likely to cross open areas (i.e., rivers or open habitat between forest refugia) and therefore more susceptible to diversification across historic barriers than are more vagile species (e.g., Develey and Stouffer 2001; Laurance *et al.* 2004; Castellon and Sieving 2006; Lees and Peres 2009; Kahindo *et al.* 2017).

Also, comparisons between the Lower Guinean and Congo Forest blocks, as well as intra-block comparisons between areas from Nigeria/Cameroon to the DRC, reveal substantial levels of genetic divergence that may be associated with more than one mechanism. These regions contain several major rivers such as the Sanaga (Cameroon), Ogooué (Gabon), and Congo Rivers, all of which have been implicated as barriers for vertebrate taxa. For example, the Congo River was recently found to be a barrier for four of 10 avian species; these four were understory lineages (Voelker *et al.* 2013). Yet

overall, investigations of the possible influence of Afro-tropical rivers on the gene flow of avian species is lacking (but see Fuchs and Bowie 2015), especially of dispersal-limited understory species. However, evidence has been recovered for the Sanaga and Ogooué Rivers as possible genetic barriers in mammalian taxa (Tefler *et al.* 2003; Querouil *et al.* 2003; Anthony *et al.* 2007; Nicolas *et al.* 2011; 2012), many of which may show similar avoidance of open spaces as understory birds. This suggests the need to assess Guineo-Congolian rivers as possible contributors to complex regional diversification patterns.

Overall, our results suggest that the Guineo-Congolian lowland tropical forests of Africa are simultaneously acting as an "evolutionary cradle" and an "evolutionary museum". The "evolutionary cradle" scenario describes tropical forests as centers of diversification, and our analyses clearly indicate high levels of diversification across a host of avian species. The large number of substantial divergences between multiple regions of the Guineo-Congolian lowland forest suggests that significant avian diversity has been created *in situ* over the past 3 - 5 million years. This strongly implies that lowland forests have served as diversification centers for numerous lowland avian species, likely due to repeated, widespread landscape alterations in the Plio-Pleistocene.

Alternatively, the "evolutionary museum" concept describes lowland forests as housing "ancient", often widespread species lacking deep geographically-structured genetic variation. Our results also support this scenario as, despite repeated habitat fragmentation, some species show little genetic diversification across their range. Since the majority of these species are dispersive taxa, it appears likely that higher levels of

vagility allow them to disperse across fragmented landscapes, thus maintaining high levels of gene flow through time. The conclusion that Guineo-Congolian forests are both diversification centers and slow zones, dependent upon species, is consistent with other studies which concluded that tropical regions may act as both "evolutionary cradles" and "evolutionary museums" in an array of taxa (Jablonski *et al.* 2006; McKenna and Farrell 2006; Moreau *et al.* 2013).

CHAPTER VI

CONCLUSIONS

Highlighting the importance of documenting diversity patterns are the multitude of destructive pressures faced by Guineo-Congolian forests. Our understanding of the various drivers leading to deforestation or habitat degradation is far from complete (Mosnier *et al.* 2014), but recent studies have shown that deforestation in the Afro-tropics has been increasing, likely due to rising human populations (Ernst *et al.* 2012; Aukema *et al.* 2017). Specifically, the lowland forests of Central Africa have maintained a relatively low deforestation rate, yet studies predict a rise in habitat destruction as human populations and political stability increase in the region (Mosnier *et al.* 2014). On the other hand, the Upper Guinean tropical forests, traditionally considered a major biodiversity hotspot (Brooks *et al.* 2002), may be a more immediate conservation imperative, as less than 80% of the original forests remain unaltered and only ~5% are under strict protection (Norris *et al.* 2010).

Since 1997, the "evolutionary museum" concept has been the dominant paradigm regarding avian diversification in the Guineo-Congolian lowland forests. The idea that these forests have largely been uneventful evolutionary regions during much of the Plio-Pleistocene and therefore house many widespread, uniform species has seemingly influenced the direction of research over the past two decades. Most studies interested in the evolutionary history of Afro-tropical avian species focused on the montane forests of East Africa, described by the Montane Speciation Hypothesis as the centers of historic

avian diversification. However, our data clearly indicate that the Guineo-Congolian forests are housing high levels of intra-specific avian genetic diversity, much of it cryptic (i.e., not reflected in phenotype), at a level entirely contrary to an "evolutionary museum" concept. This concept focused on relatively old species (> 5 Ma) described entirely by morphology with little subsequent attention given to exploring the possibility of cryptic lineages being imbedded within them. Indeed, one genus of Afro-tropical birds (*Stiphrornis*) that has seen multiple, rigorous investigations (i.e., genetics, morphology, behavior) was originally described as one widespread species lacking substantial plumage variation and then shown to contain no less than eight species (Beresford and Cracraft 1999; Schmidt *et al.* 2008; Voelker *et al.* 2016). Given the substantial divergences found in our analyses, coupled with a lack of rigorous phylogenetic focus on the vast majority of lowland forest birds (many of which are widespread and lack discrete plumage variation), it seems exceptionally likely that numerous cryptic species or lineages have yet to be discovered.

Furthermore, cryptic diversity in Afro-tropical lowland forests isn't restricted to birds, but has been demonstrated in mammals (Nicolas *et al.* 2005, 2008, 2011, 2012; Anthony *et al.* 2007; Jacquet *et al.* 2015; Gaubert *et al.* 2016), reptiles (Portillo and Greenbaum 2014; Greenbaum *et al.* 2015; Portillo *et al.* 2015) and even invertebrates (Voelker *et al.* 2013; Light *et al.* 2016; Takano *et al.* 2017). Clearly, we have scientifically underestimated the amount of biodiversity present within the Guineo-Congolian lowland forests of Africa. This consistent underestimation of diversity can lead to a flawed understanding of how to protect species and the areas they occupy

(Pimm *et al.* 2014). Many conservation organizations have proposed schemes centered on scrutinizing irreplaceability (endemism), species richness, and vulnerability when prioritizing regions of concern around the globe (Aukema *et al.* 2017). The data we present here suggests that we may have little understanding of (i) which areas of the forest are vital for creating and maintaining biodiversity, (ii) true avian species limits, and (iii) the full extent of diversification patterns across the Guineo-Congolian forests. Our lack of understanding may be causing us to underestimate both irreplaceability and species richness across the region, leading to a flawed paradigm of conservation

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