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MORPHOLOGICAL AND GENETIC VARIABILITY IN GHANAIAN POPULATIONS OF NOACK'S LEAF-NOSED BAT (*HIPPOSIDEROS* AFF. *RUBER*) ALONG A LATITUDINAL GRADIENT

BY

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DECLARATION

I hereby declare that this thesis is the result of my own research conducted under supervision and that no part of it has been published or presented for an award of a degree elsewhere, except for references from other sources, that have been duly acknowledged.

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ABSTRACT

The *Hipposideros caffer* complex consists of several taxa whose taxonomic relationships are not fully resolved. Genetic diversity and skull morphology of Ghanaian populations of the Noack's leaf-nosed bat *Hipposideros* aff. *ruber* (Noack, 1893) attributed to mitochondrial D lineages were investigated using nucleotide sequences of mitochondrial *cytochrome b* gene and geometric morphometrics. *Cytb* sequences of *H.* aff. *ruber* populations from Ghana were compared to other available West African sequences and their phylogenetic relationships were inferred under maximum likelihood, maximum parsimony and Bayesian methods. Phylogenetic reconstruction based on 63 *cytb* sequences (725 base pairs) showed a paraphyly in the Ghanaian populations. Individuals from the southern part of Ghana (Kwamang, Buoyem and Forikrom sites) formed a monophyletic group with individuals from Cote d'Ivoire, which corresponds to the previously identified sublineage D3. On the other hand, individuals from the northern part of Ghana clustered together with individuals from Senegal and Benin within sublineages D1 and D2, respectively. Interestingly, the Ghanaian populations exhibited a rather large genetic divergence of up to 6.1% based on pairwise Kimura two-parametric distances. This suggests possible presence of cryptic species, but reproductive isolation is yet to be investigated to confirm species status of the divergent genetic forms. Differences in size and shape of skull morphology of the D lineage were investigated using 10, 11, and 13 digitized landmarks on dorsal, ventral and lateral views respectively. Intra-lineage shape differences analyzed by Canonical Variate Analysis (CVA) and Discriminate Function Analysis (DFA) with leave-one-out cross validation of the Procrustes superimposed landmarks showed variation in the region of the zygomatic arch. Individuals assigned to sub-lineages D3 are distinguished from the other two sub-lineages (D1, D2) by having a slightly wider zygomatic arch. Cross-validated classification results were greater than 70% for sub-lineages D1 and D3 as well as D2 and D3 for all three cranial views analyzed but were as low as 47% when comparing D1 and D2. Classification success for the three cranial views for northern and southern Ghana populations ranged from 68%-85%. Log centroid size differed among lineages, sexes as well as northern and southern Ghana populations. Mechanisms of isolation by distance and historical environmental changes may have played a major role in the evolution of the D sub-lineages. Broader geographical sampling throughout West Africa and the use of both nuclear and mitochondrial data are necessary for resolving the phylogeography of the complex.

DEDICATION

I dedicate this work to the Opoku family particularly to Mr. Matthew Akwasi Opoku, Agnes Opoku, Georgina Donkor and all committed to the conservation of Mother nature.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Bats are the second most numerous mammalian group after rodents, exhibiting both unique evolutionary characteristics and adaptations (Wilson & Reeder, 2005; Saikia, 2007). This successful adaptive radiation of bats relative to other mammalian groups is attributed to both true flight and echolocation abilities (Kunz & Pierson, 1994; Kalko & Schnitzler, 2001). Bats belong to the order Chiroptera which is divided into two suborders; Yinpterochiroptera and Yangochiroptera (Teeling *et al*., 2005). Insectivorous bats regulate both agricultural pests and disease vectors such as mosquitoes (Rydell *et al*., 2002; Boyles *et al*., 2011) whereas fruit-and-nectar feeding bats provide both seed dispersal and pollination services (Hodgkison *et al*., 2003). Such ecosystem services are indicative of the value of bats to the food production systems and general well-being of humans.

A fundamental challenge in biological research is the identification of organisms to meaningful taxonomic levels. Species are considered cryptic if two or more morphologically similar species are traditionally assumed to be a single species but are separable based on other lines of evidence such as genetics or behaviour (Bickford *et al*., 2007). Recovery of cryptic species within traditionally recognised groups poses a basic question of how effectively organisms can be identified, used and conserved. It presents serious challenges for management of natural resources and biodiversity conservation by questioning the fundamentals of biodiversity estimates, extinction risk assessment protocols and disease and invasive pest management strategies (Paterson, 1991; Bickford *et al*., 2007; Hu *et al.*, 2014). It also casts doubts on the relevance of current conservation action plans if one or more of the newly-recognised species is vulnerable to the risk of extinction (Schonrogge *et al*., 2002). Recently, cryptic bat species have been recovered from many traditionally-recognized groups based on molecular, acoustic, behavioural and morphological analyses (Kingston *et al*., 2001; Thabah *et al*., 2006; Vallo *et al*., 2008; 2011; Koubínová *et al*., 2013).

Traditionally, the leaf-nosed bat, *Hipposideros caffer* has been regarded as a complex of populations. The Noack's leaf-nosed bat *Hipposideros ruber* (Noack, 1893) is one of the currently recognized cryptic species within the Sundevall's leaf-nosed bat *Hipposideros caffer* (Sundevall, 1846) complex. It was considered as two cryptic species by Hayman and Hill (1971) but currently understood to consist of four major phylogenetic groups representing at least five species in Africa (Vallo *et al*., 2008). In Ghana, three distinct mitochondrial (mtDNA) lineages (labeled B, C and D) morphologically pertaining to *H. ruber* have been previously confirmed, which can be considered as separate species. Hence, they are all referred to as *H.* aff. *ruber* until formally acknowledged by the International Commission on Zoological Nomenclature. One of the mtDNA lineages, the so-called D-lineage *sensu stricto* Vallo *et al*. (2008) is particularly common in central Ghana. This D-lineage has been confirmed also from Senegal and Benin, however, with a considerable genetic distance (2.0-5.3%) to the population of southern Ghana (Vallo *et al*., 2008; 2011).

1.2 Problem Statement and Justification

Conventionally, the identification of members of the genus *Hipposideros* (Gray, 1831) has been difficult due to phenotypic similarities of most of its members (Hill, 1963; Bogdanowicz & Owen, 1998; Vallo *et al*., 2011). Thus, the systematics of the African *Hipposideros* taxa remains unresolved even with the recovery of cryptic species based on molecular genetics (Vallo *et al*., 2008; 2011; Monadjem *et al*., 2013). Vallo *et al*. (2008) showed that although populations of *H*. aff. *ruber* from Senegal and Benin are distantly separated, they are evolutionarily more closely-related to each other than each of them to the population of central Ghana. Yet, it remains largely unknown if there are any morphological differences between *H*. aff. *ruber* populations in southern and northern Ghana and whether populations of northern Ghana are evolutionarily more closely related to the southern Ghanaian woodland populations or the savanna populations from Senegal and Benin.

Majority of studies on *H*. aff. *ruber* in Ghana have concentrated on the potential of the species as a possible host of emerging infectious diseases with zoonotic potential (Pfefferle *et al*., 2009; Drexler *et al*., 2012; Annan *et al*., 2013). Given the challenges posed by the discovery of cryptic species, erroneous identification of economically- and medically-important species such as bats may have serious implications for both human health and agriculture. The *H*. aff. *ruber* complex in Ghana, for example, acts as host to a number of potential zoonotic diseases. Misidentification of such a species could render established disease preventive and control protocols ineffective. Habitat changes resulting from the continuous conversion of the isolated forest fragments to farmlands and other human dominated land use systems in Ghana (Appiah *et al*., 2009) reemphasize the need for studying phylogenetic relationships among local fauna. This is particularly useful for understanding evolutionary processes, ecology, biogeographic principles operating at the local scale and perhaps recovery and conservation of rare species (Koubinova, 2013). Further, identification to a meaningful taxonomic level (species) may also provide new useful research avenues for understanding ecological interactions that exist among sympatric species. Thus, it is imperative to carry out both morphometric and molecular phylogenetic analysis on *H.* aff. *ruber*.

While species from the D-lineage of Senegal have been confirmed to be morphologically similar based on traditional morphometric methods (Vallo *et al*., 2011), geometric morphometric studies on *H.* aff. *ruber* populations from Ghana and the sub-region are generally absent. Traditional morphometric methods (TM) have been criticized for sometimes being inadequate for differentiating cryptic species in contrast to geometric morphometric methods (GMM) (Evin *et al*., 2008; Sztencel-Jabłonka *et al*., 2009; Schmieder *et al*., 2015). Traditional methods involve both univariate and multivariate statistical analyses on matrices of quantitative variables such as lengths, ratios and angles which were consistently inadequate as morphometric (Marcus, 1990; Adams *et al*., 2004; 2013). Information on the geometric relationships of organisms were not adequately preserved throughout the analysis since most measurements originated from single non-homologous points which were also highly correlated with size. It provided inadequate information about shape changes and graphical illustrations were difficult (Adams *et al*., 2004; 2013; Zelditch *et al*., 2012). Thus, traditional morphometric methods may not allow taxonomists to fully comprehend morphological variability that may exist among and within populations. Further, the existence of overlaps in commonly used morphological characteristics such as forearm length or condylocanine length (Hayman & Hill, 1971; Koopman, 1975; Vallo *et al*., 2011) emphasizes the overall urgency of the need for adequate diagnostic identification features.

Conversely, GMM permits precise detailed descriptions, separation of size and shape components, accurate statistical analysis while allowing for visualization of morphological changes among organisms. It is based on the use of Cartesian coordinates of discrete anatomical loci, the so-called landmarks to characterise organismal shape changes (O'Higgins, 2000; Zelditch *et al*., 2012; Adams *et al*., 2013). Such detailed analysis of morphological characters may provide better diagnostic identification features for differentiating between cryptic species which may further provide useful insights for the resolution of the systematics of the *H.* aff. *ruber* complex.

Skull and mitochondrial DNA (mtDNA) were used for the study of both morphological and molecular genetic diversity respectively, of bats. In morphological studies, the skull represents an important structure for studying both ontogenetic and functional processes. It provides insights into both ecological and evolutionary processes (Adams & Pedersen, 2000). Predictions on diet, for example, have been made based on craniodental morphology (e.g. Freeman, 1979; Ghazali & Dzeverin, 2013). Mitochondrial DNA (mtDNA) is the most widely-used marker for inferring evolutionary history among several taxa due to its numerous advantages (Avise, 2000; Baker & Bradley, 2006). It is predominantly maternally-inherited, evolves rapidly, diploid, typically non-recombining and relatively easy to amplify using the polymerase chain reaction (PCR) procedure. Therefore, it is useful for simplified reconstruction of within species evolutionary history, delineating divergence times and inferring population structure among species (Ballard & Whitlock, 2004; Galtier *et al*., 2009).

1.3 Objectives

The general objective of the study was to investigate the morphological and genetic variations of *H.* aff. *ruber* populations in Ghana in relation to other West African forms in order to provide adequate diagnostic features for proper identification of the species. The specific objectives were to:

- assess morphological variation among populations of *H.* aff. *ruber* from Ghana using geometric morphometric analysis of skulls.
- assess phylogenetic relationships within the Ghanaian *H.* aff. *ruber*

1.4 Hypothesis

Based on the supposed phylogenetic relationship of *H.* aff. *ruber* (D-lineage) from West Africa, it is hypothesized that populations from northern Ghana will be more evolutionarily closer to populations from Benin and Senegal than those from southern Ghana

CHAPTER TWO

LITERATURE REVIEW

2.1 Phylogeny and Systematics of Bats

Phylogenetic inference is based on the concept of homology, or comparison of shared characters of common ancestral descent (Fitch, 1970). It is useful for inferring recent and past evolutionary, ecological and biogeographical history even in the absence of fossils while providing useful insights into morphological, behavioural or organismal changes over time (Avise, 2004; Russell *et al*., 2008). Conventionally, evolutionary relationship among species have been largely studied based on comparison of morphological characters (e.g. Simmons & Geisler, 1998). However, many traditionally recognized groups have not been confirmed using molecular phylogenetic studies. A classic example is the common pipistrelle bat, *Pipistrellus pipistrellus* (Schreber, 1774) which was traditionally considered to be monotypic but are now considered as two distinct species *P. pipistrellus* and *P. pygmaeus* based on mtDNA sequences (Barrat *et al*., 1997).

Traditionally, the order Chiroptera forms a monophyletic sister taxon subdivided into Megachiroptera and Microchiroptera on the basis of morphology and paleontological studies. Megachiroptera consists of one family, Pteropodidae, characterized by the use of vision while Microchiroptera consists of all other known bat families using laryngeal echolocation (Koopman, 1994; Simmons & Geisler, 1998). This conventional view was challenged by the 'flying primate hypothesis' which suggests that megabats are phylogenetically closer to primates than microbats, making bats diphyletic (Pettigrew, 1986; Pettigrew *et al*., 1989). Accordingly, on the basis of neuroanatomical features, bats are grouped into the superorder Archonta of the class Mammalia together with the orders Scandentia, Dermoptera and Primates.

Recent molecular phylogenetic studies provide evidence for the traditional monophyly of bats (Ammerman & Hills, 1992; Nikaido *et al*., 2000; Teeling *et al*., 2000) but introduce a contrasting hypothesis that megabats are evolutionarily closely-related to microbats in the superfamily Rhinolophoidea in contrast to microbats, thus suggesting a polyphyletic clade (Hutcheon *et al*., 1998; Teeling *et al*., 2000). However, several analytical criticisms refuted this hypothesis (reviewed in Jones *et al*., 2002). Pettigrew (1986) argued that strong associations of many morphological characters used in cladistic studies with flight and metabolic constraints imposed by flight may confound the monophylic or diphylic grouping of bats. DNA base compositional bias resulting from excessively shared proportions of adenine (A) and thymine (T) in the DNA of megabats and some microbat families may bias support towards the monophyly of bats (Pettigrew, 1994; Pettigrew & Kirsch, 1995). Lastly, the adequacy of the level of taxonomic sampling of molecular studies to reject bat monophyly has been questioned given that long-branch attraction (Swofford & Olsen, 1990) may account for the unification of both mega- and microchiropteran clades (Kirsch & Pettigrew, 1998; Pettigrew & Kirsch, 1998).

Presently, bats are grouped in the superorder Laurasiatheria in contrast to Achonta and are not a sister taxon to Dermoptera (Madsen *et al*., 2001; Murphy *et al*., 2001; Teeling *et al*., 2002). The monophyly of extant microbats has been largely rejected for a paraphyletic group based on molecular studies. The order Chiroptera is divided into two suborders, Yinpterochiroptera (Pteropodidae and Rhinolophoidae) and Yangochiroptera (other microbats) (Teeling *et al*., 2000;

2002, Springer *et al*., 2001). In view of this, four major superfamilies of echolocating microbats have been identified: (i) Rhinolophoidea (rhinolophids, hipposiderids, rhinopomatids, craseonycterids, megadermatids), (ii) Emballonuroidea (nycterids and emballonurids), (iii) Vespertilionoidea (vespertilionids, molossids, natalids, miniopterids) and (iv) Noctilionoidea (noctilionids, phyllostomids, mormoopids, furipterids, thyropterids, mystacinids, myzopodids) (Figure 1) (Teeling *et al*., 2012).

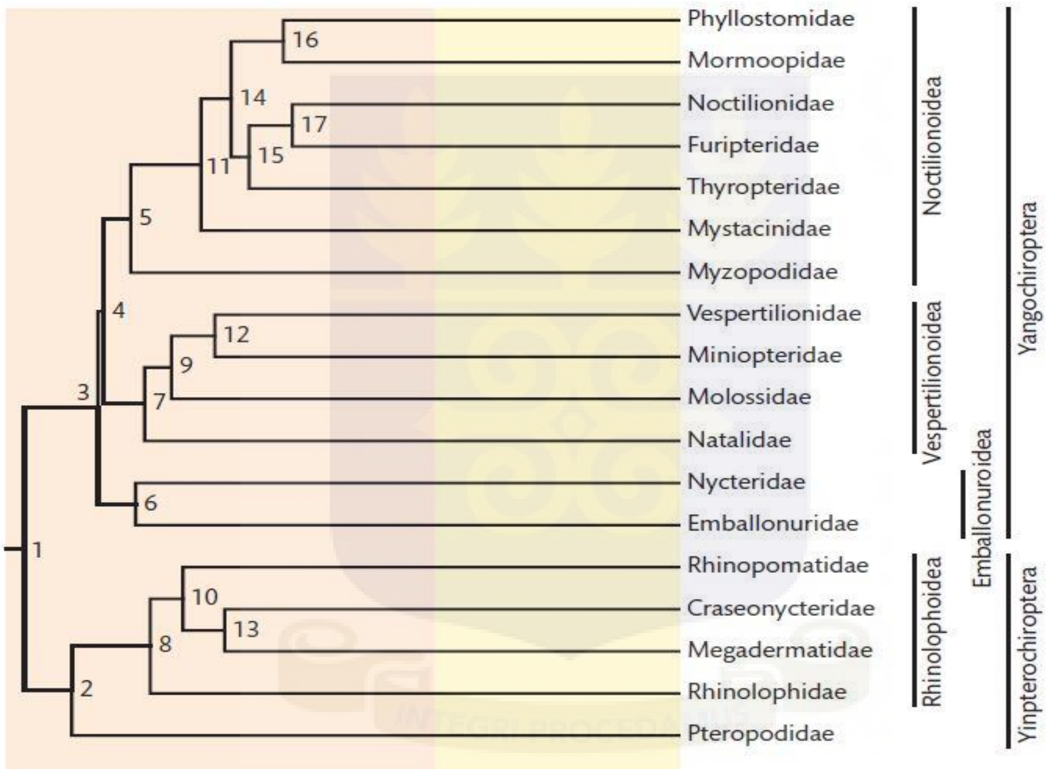

Figure 1: Phylogenetic relationship among bats. Adapted from Teeling (2009).

2.2 Family: Hipposideridae

The family of leaf-nosed bats, Hipposideridae (Lydekker, 1891) is a polytypic family with nine extant genera extensively distributed in tropical and subtropical regions of the Old World (Simmons, 2005; Happold & Happold, 2013). The genus name *Hipposideros* is derived from the Greek words “hippos” (horse) and “sideros” (iron) to describe the horseshoe-like appearance of the nose leaf (Rosevear, 1965). Conventionally, it was grouped as a subfamily of Rhinolophidae by several authors including Simmons & Geisler (1998) and Teeling *et al*. (2002) but was returned to family rank by Bogdanowicz & Owen (1998) and Simmons (2005).

The genera *Anthops* (Thomas 1888), *Paracoelops* (Dorst 1947), *Rhinonycteris* (Gray 1847) and *Cloeotis* (Thomas 1901) are monotypic; *Coelops* (Blyth, 1848) has two species; *Asellíscus* (Tate 1941) and *Asellia* (Gray 1838) have three species; *Triaenops* (Dobson 1871) has six species and *Hipposideros* (Gray, 1831) has over 70 species (Bogdanowicz & Owen, 1998; Simmons, 2005; Bates et al., 2007; Benda et al., 2011; Thong et al., 2012). Benda & Vallo (2009) have suggested a new genus, *Paratriaenops* for Malagasy species, *Triaenops furculus*, *T. auritus* but *T. menamena* and one species from western Seychelles, *T. pauliani* formerly grouped in *Triaenops*. The genera *Triaenops*, *Paratriaenops* and possibly *Cloeotis* have been split from other hipposiderids of the tribe Hipposiderini and included in the tribe Triaenopini. Tate (1941) recognised that the genus *Hipposideros* comprised of loosely-defined groups exhibiting different combinations of characteristics and divided the genus into 11 species groups without detailed consideration of its African species. Seven species groups were subsequently recognised by Hill’s (1963) revision of the genus based on different levels of character development within *Hipposideros*: *megalotis, bicolor, cyclops, pratti, armiger, diadema* and *speoris* groups.

2.3 *Hipposideros caffer* Complex

Bats in the *Hipposideros caffer* complex fall into the *H. galeritus* subgroup within the *bicolor* group with other African congenerics: *H. beatus* (Andersen 1906), *H. fuliginosus* (Temminck, 1853), *H. curtus* (Allen 1921) and *H. lamottei* (Brosset, 1985). Subsequently, the complex was considered as a single species, *H. caffer* (Hill, 1963), two species, *H. caffer* and *H. ruber* (Hayman & Hill, 1971; Simmons, 2005) but currently consist of four major phylogenetic groups (labelled A, B, C, D) representing at least five species in Africa (Vallo *et al*., 2008).

Kimura two-parameter (K2P) genetic distances within the African *H. caffer complex* are 11.3% and up to 20.4% in comparison to other species (Vallo *et al*., 2008). Varied genetic distances have been documented for cryptic Asian hipposiderids: 3.9–4.1% among *H. khakhouayensis* and *H. rotalis* (Guillén-Servent & Francis, 2006), 6.5-6.8% among 142 kHz *H. bicolor* and 131kHz *H. bicolor* (Kingston *et al*., 2001), 12-13.4% among *H. khasiana* and *H. grandis*, (Thabah *et al*., 2006). According to Vallo *et al*. (2008) lineage A, which differed from other lineages by a genetic distance of 7.4–11.3%, corresponds to *H. caffer* morphotypes from Southern Africa whereas representative samples of the sister lineage A2 from Morocco, Senegal and Yemen correspond to the subspecies *H. caffer tephrus*. Lineage B consisted of both *H. caffer* and *H. ruber* specimens, separated into two subclades B1 and B2 by a genetic distance of 5.1–7.0%. Monadjem *et al*. (2013) recovered sister relationships between *H. lamottei* from Mount Nimba and Vallo *et al*. (2008) western African subclade B1 and eastern African subclade B2 with sequence divergence of about 6%.

Lineages C and D corresponded to *H. ruber* morphotypes from Central and West Africa respectively. Both lineages may represent cryptic species given a genetic distance of 4.2-6.6% between sister lineages C1 and C2 and a large divergence of lineage D, 7.5–11.0% from other lineages (Vallo *et al*., 2008). Monadjem *et al*. (2013) recovered 2 lineages E1 and C1 separated by a genetic distance of over 9% which corresponded to lineages D1, D2 and sub-clade C1 of Vallo *et al*. (2011, 2008) respectively. Members of these two phylogroups emit different echolocation calls ranging from 127-128 KHz for E1 and 145-150 KHz for C1 which are distinguished from *H. lamottei* calls within the range 118-120 KHz. Further, it has been suggested that individuals of lineages E1 and C1 are morphologically similar to morphotypes of *H. caffer centralis* (Andersen, 1906) from Uganda and *H. caffer guineensis* (Andersen, 1906) from Gabon, respectively. However, their systematics remain unresolved (Monadjem *et al*., 2013).

The distinct West Africa D lineage from Senegal, Cote d'Ivoire, Ghana and Benin differed by a genetic distance of 2.0-5.4% (Figure 2). Specimens from Senegal clustered in lineage D1 and D2 in sister relationship with specimens from Benin whereas specimens from Cote d'Ivoire and Ghana also group as sisters (Vallo *et al.*, 2011). According to Monadjem *et al*. (2013), lineages D1 and D2 from Senegal form sister relations with specimens from Ghana, Liberia and Cote d'Ivoire but the sister relationship between these two lineages was not supported. The discrepancy between geographically distant populations from Senegal and Benin with a genetic distance of 2% and geographically close populations of Ghana and Benin with over 5% differences suggest the need for further sampling of *H.* aff. *ruber* populations to elucidate the systematics of the complex (Vallo *et al*., 2011).

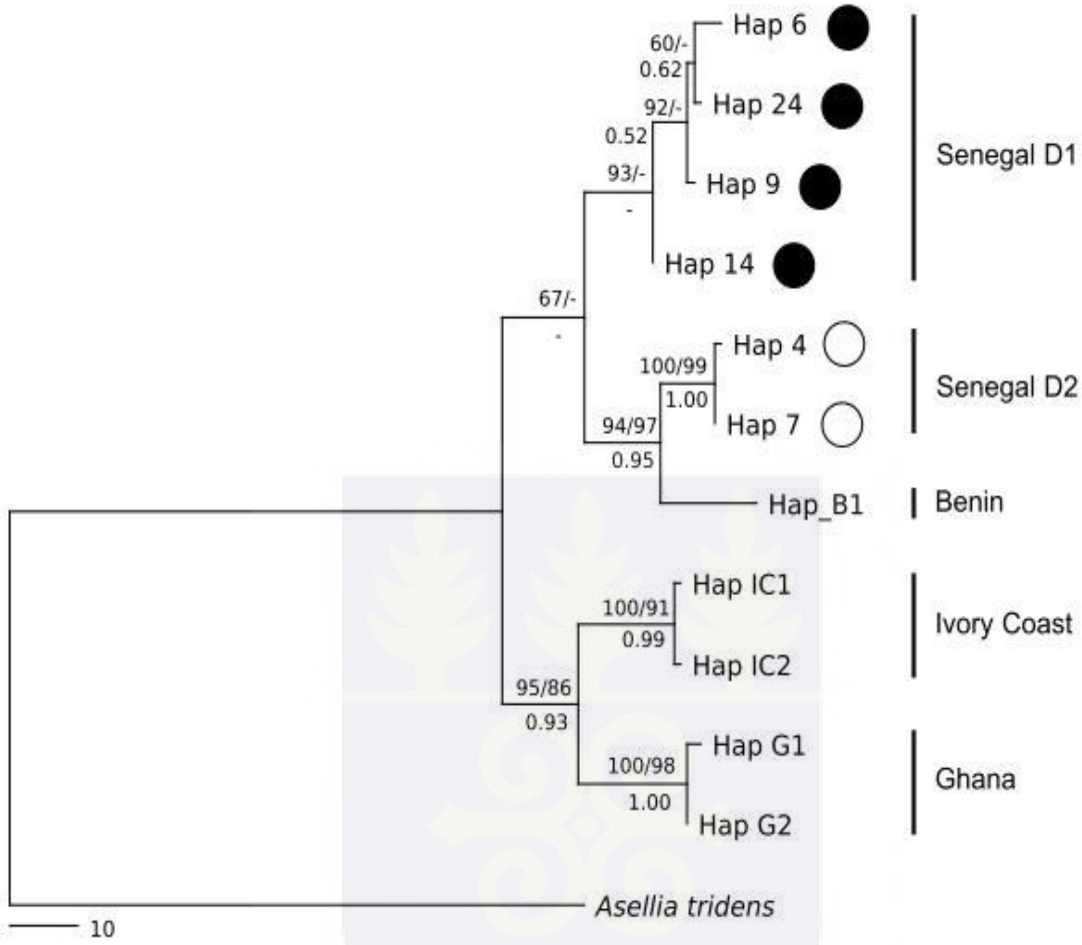

Figure 2: Phylogenetic relationships among West African *H.* aff. *ruber*. Adapted from Vallo *et al*. (2011).

2.4 Study Species: *Hipposideros* aff. *ruber*

Hipposideros aff. *ruber* represents possibly a separate species within the *H. caffer* complex. *Hipposideros ruber* is about 87.5 mm long (total body length), has a forearm length of about 47-55 mm, with a wingspan of about 308 or 320 mm and a body mass of about 7-12g., while *H. caffer* has an average body length of 78.4 mm, a forearm length of about 42-52 mm, with an average wingspan of 288 mm and a body mass of about 5-11g (Happold & Happold, 2013). *Hipposideros ruber* has a relatively large but short and broad skull with zygomatic width greater than or less than the mastoid width whereas *H. caffer* has a short, broad skull with zygomatic width equal to, greater than or less than the mastoid width. Both forms have a low sagittal crest and relatively small cochlea (Hill, 1963; Happold & Happold, 2013).

Modifications in the shape of nasal swelling compartments are important distinguishing features between these two forms due to their influence on nasal echolocation (Lawrence, 1964). Both forms possess two leaflets lateral to the nose leaf, visible nostrils with low and small internarial septum and no club-shaped processes on the central nose leaf (Rosevear, 1965; Happold & Happold, 2013). *Hipposideros caffer* has small rounded nasal swelling compartments and large posterolateral nasal swelling compartments relative to *H. ruber* (Lawrence, 1964; Koopman, 1975). In *H. ruber*, the median posterior narial region is relatively wide with narrow lateral inflations as compared to a narrow median posterior narial component with wide lateral inflations in *H. caffer* (Happold & Happold, 2013). Generally, both forms show no sexual dimorphism but display two colour phases. The fur of *H. ruber* is more rufous in color and more brownish whereas *H. caffer* is orange and more grayish during the bright and dull colour phases respectively (Hayman & Hill, 1971). Frontal sacs are present in both sexes of *H. caffer* but only in males of *H. ruber* (Happold & Happold, 2013).

Hipposideros caffer has a widespread distribution in Africa particularly south of the Sahara and in south-western Arabian Peninsula except the central forested region and in Morocco, Zanzibar and Pemba. It occurs commonly in woodland savanna, arid ecosystems and seasonally dry open forests of its range but absent from the more arid regions and the primary rainforest belt (Hayman & Hill, 1971; Simmons, 2005). *Hipposideros ruber* ranges in lowland rainforest and woodland savanna throughout Africa, Sao Tome and Principe but is absent from the drier southernmost and eastern regions of the continent. It has been recorded from Gambia, Senegal to Ethiopia and south of Angola, Malawi and Mozambique (Happold & Happold, 2013). Both forms occur in sympatry in open forests and savannas surrounding the rainforest belt (Hayman & Hill, 1971; Simmons, 2005).

Conventionally, *H. ruber* and *H. caffer* (Plate 1) are identified based on use of morphological (forearm length and condylocanine length) and echolocation call parameters. Generally, *H. caffer* is smaller than *H. ruber* (Hayman & Hill, 1971; Koopman, 1994) and emits a higher frequency of the echolocation call (Heller, 1992; Jones *et al*., 1993). *Hipposideros caffer* has forearm lengths smaller than 48 mm and condylocanine length less than 15.5 whereas *H. ruber* has forearm lengths more than 48 mm and condylocanine length greater than 15.5 (Hayman & Hill, 1971; Koopman, 1975). Nonetheless, variations in morphological measurements have rendered these identification keys insufficient for separating both forms (Heller, 1992; Vallo *et al*., 2011).

Both forms use echolocation frequency calls of both constant frequency (CF) and frequency modulated (FM) components for orientation and foraging (Schnitzler & Kalko, 2001). *Hipposideros ruber* emits echolocation call of about 130-140 kHz (Heller, 1992) with little geographical variation while *H. caffer* emits variable calls with respect to location. Call frequencies of 130–160 kHz have been recorded for populations from Nigeria, Kenya, and Uganda (Pye, 1972). Jones *et al*. (1993) and Heller (1992) also recorded call frequencies of 128-153 kHz and 155-157 kHz for *H. caffer* from West Africa and DR Congo respectively. Such variations in call frequency in different locations suggest the existence of cryptic species (Happold & Happold, 2013).

Hipposideros ruber and *H. caffer* forage in different habitat types but use slow-hawking and gleaning strategies during feeding (Bell & Fenton, 1984; Happold & Happold, 2013). *Hipposideros caffer* has a variable seasonal diet but feeds predominately on moths (Bell & Fenton, 1984; Dunning & Krüger, 1996). *Hipposideros ruber* feeds mostly on beetles in cluttered habitats (Aldridge & Rautenbach, 1987; Jones *et al*., 1993). The most recent review on *H. caffer* is provided by Wright (2009) and bats in general by Happold and Happold (2013).

A B

Plate 1: *Hipposideros* aff. *ruber* from Mole National Park (A) and Kwamang (B)

(Source: Fieldwork)

2.5 Traditional and Geometric Morphometrics

Morphometrics refers to the study of shape variation and its covariation with other variables (Bookstein, 1991). Morphological variability among organisms were traditionally investigated by statistically analysing sets of quantitative variables corresponding to morphological and anatomical features (Marcus, 1990; Adams *et al*., 2004). These variables included measurement of linear distances, ratios, angles and counts which were analysed by both univariate and multivariate analyses. Principally, multiple analysis of variation (MANOVA), multiple analysis of covariation (MANCOVA), principal components analysis (PCA), factor analysis, canonical variates analysis (CVA) and discriminant function analysis have been used in quantifying size and shape changes among biological species (Adams *et al*., 2004, 2013).

Traditional morphometric methods (TM) are limited in their ability to provide biological interpretation of shape as they do not fully capture and retain the geometry of the morphological features of organisms under study. Most measurements were concentrated on very few specific features and as such many important features which may contribute to the overall shape changes may be under-represented (Zelditch *et al*., 2012; Adams *et al*., 2013). Further, they were not based on homologous features and were highly correlated with size thus providing little information independent of size. Another major drawback of TM was the difficulty with visually displaying changes in organismic shape. This made it difficult to assign biological interpretation to results that were mostly presented in a variable-covariable matrix (Bookstein *et al*., 1985; Adams *et al*., 2004, 2013). Geometric morphometric methods (GMM) quantify changes in shape of biological organisms using the Cartesian coordinates of discrete anatomical loci, landmarks. These landmarks, either two- or three-dimensional, are homologous and provide sufficient coverage of the morphology and are readily repeatable. (Bookstein, 1991; Zelditch *et al*., 2012). Thus, the geometry of the organism is preserved throughout the study and allows for visualization of shape changes.

According to Kendall (1977), shape is “all the geometric information that remains when location, scale and rotational effects are filtered out from an object”. All non-shape variables are removed from the geometric analysis of shape by superimposition of the landmark configuration via some optimization criterion. A generalized Procrustes analysis (GPA) based on a least-squares procedure is used to generate the mean shape and project the landmark configurations into a linear tangent space for further multivariate analysis (Rohlf & Slice, 1990; Rohlf, 1999; Kent & Mardia, 2001; Adams *et al*., 2013). This least-squares method removes the effect of location, size and rotation by translating all configuration matrices to the centre, rescaling to a unit centroid size and rotating to minimize the squared difference between the corresponding landmarks (Adams *et al*., 2004, 2013). Multivariate statistical testing of the biological hypothesis and visualisation of shape changes among and within species is possible after the mean shape is generated. For example, the Hotelling’s T^2 is useful for testing shape differences among species that are sexually dimorphic. Alternatively, an interpolation function such as a thin-plate spline technique (Bookstein, 1989; 1991) which produces a set of bending-energy matrices may be used to generate deformation grids for visual display of shape changes and for multivariate analysis (Rohlf, 1998; Zelditch *et al*., 2012).

2.6 Morphology and Echolocation

Currently, two competing hypotheses on the evolution of echolocation in bats are recognized based on microbat paraphyly. Based on both molecular and paleontological evidence, Springer *et al*. (2001) postulate that echolocation was present in all bats but subsequently got lost in megabats and re-evolved in the genus *Rousettus.* Contrarily, Eick *et al*. (2005) argue that echolocation could have evolved separately in common ancestors of Yinpterochiroptera and Yangochiroptera based on the presence of unique synapomorphy associated with echolocation in Rhinolophoidea. However, analyses of cochlear morphology support the hypothesis that laryngeal echolocation in extant bats evolved subsequent to the divergencies of the chiropteran suborders (Davies *et al.*, 2013).

All echolocating bats (microbats) use vocal chord-based biosonar vocalizations except the cave-dwelling Egyptian fruit bat, *Rousettus aegyptiacus* (megabat) that uses tongue-generated clicks for gathering information on the environmental features, characteristics of potential prey items and orientation in space (Kalko & Schnitzler, 2001; Waters & Vollrath, 2003; Holland *et al.*, 2004). Microchiropterans emit calls made of narrowband or broadband components or both, due to offset between prey identification and precision of localisation. Quasi-constant frequency (QCF) and long constant frequency (CF) elements constitute the narrowband component while the broadband component is made up of the downward frequency-modulated (FM) component of large bandwidth (Schnitzler & Kalko, 2001). Most members of Rhinolophidae, Hipposoderidae, Emballonuridae, Molossidae, Mormoopidae, Vespertilionidae and Noctilonidae use intense echolocation calls (>110 dB SPL) to hunt prey during foraging (Bogdanowicz *et al.*, 1999). Within these families, two approaches to echolocation are distinguished based on the amount of time a signal is 'on', the so-called duty cycles. Low duty-cycle echolocating bats use short (0.5-30 ms) echolocation pulses separated by much longer listening intervals, hence both the emitted echolocation and the returning echo are separated in time domain whereas high-to-medium duty cycle species use longer pulses (≥ 50 ms) separated by shorter intervals thus separating both outgoing pulse and incoming echo in frequency domain (Fenton *et al*., 1995).

Generally, body mass/size (forearm length) is negatively correlated with echolocation frequency parameters (frequency, duration and repetition rate) among bats. Thus, smaller bats use high frequency calls relative to larger bats (Jones, 1999; Zhang *et al*., 2000). This relationship is clearly evident in at least five bat families: Vespertilionidae, Rhinolophidae, Hipposideridae, Emballonuridae and Molossidae. Ecological factors, the mechanics of sound production, foraging strategies, limitations imposed by echolocation and prey detection have been suggested as factors contributing to such relationships (Barclay & Brigham, 1991; Jones, 1999). However, there are exceptions to this general allometric relationship, with some bats emitting either higher or lower frequency calls than their congeners irrespective of size (Jacobs *et al*., 2007).

2.6.1 Pinna and Echolocation

Differences in shape and size of the pinna dictate variation in echolocation call design to some extent. The external pinna is effective for collecting and directing sounds returning to the bat (Obrist, 1995). Directionality of the bat pinna increases gradually at frequencies above 5 KHz and there is a positive correlation between pinna dimension, directionality and interaural intensity differences (IID) among some microchiropterans, particularly rhinolophids and hipposiderids (Obrist *et al*., 1993). Davies *et al*. (2013) demonstrated that correlation exists between the length of the basilar membrane and number of cochlear turns with echolocation call parameters (minimum frequency, maximum frequency and peak frequency) for echolocating bats. Further, it has been suggested that pinna movements of some bats such as *R. aegyptiacus* correspond at least partially with the emission of echolocation signals (Holland & Waters, 2005). These relationships suggest that characteristics of both external and inner ears may be related to echolocation call design in bats.

2.6.2 Cranium and Echolocation

Echolocation bats emit calls either through the nostrils or the mouth primarily as a result of variation in cranial morphology. Nasal-emitting microchiropterans are distinguished by the rotation of the basicranium ventrally about the cervical axis, rotation of the rostra below the braincase to align nasal cavity and rotation of the lateral canals whereas in oral-emitting microchiropterans neurocranial and orofacial components are arranged one after the order. Further, differences between the indices of choanal bore, narial bore and pterygoid width related to the construction of the nasopharynx distinguishes nasal and oral emitting microchitopterans (Pedersen, 1993; 1995; 1998). However, Goudy-Trainor (2002) could not establish any significant relationship between echolocation call parameters and facial features among nasal and oral emitting bats.

2.7 Nose Leaf Structures and Wing Morphology

Nose leaf structures are prominent facial features in the Rhinolophidae, Hipposideridae, Nycteridae, Megadermatidae, Phyllostomidae, Rhinopomatidae and in a few species of Vespertilionidae (Bogdanowicz *et al*., 1997). The functions of nose leaves are not fully understood but they are known to serve different functions in different bats. They influence directionality of the emitted signal by serving as energy-focusing apparatuses in some phyllostomids (Hartley & Suthers, 1987; Vanderelst *et al*., 2010). It has been suggested that variations in nose leaf structures maybe correlated with diet and echolocation in some bats (Arita, 1990; Bogdanowicz *et al*., 1997).

The design of the bat wing provides useful predictions about flight, hunting behaviour and the general ecology of the species. The shape and surface area of the wings as described by aspect ratio (wingspan^2/wing area) and wing loading [(mass× gravitational acceleration, g)/wing area] respectively affect manoeuvrability and agility, flight speed and energetic cost during flight. Thus, flight speed and energy efficiency increase with wing loading and aspect ratio respectively but both are inversely related to manoeuvrability (Norberg & Rayner, 1987). Generally, slow-flying bats usually have long wings of high aspect ratio, long pointed wingtips and low or average wing loading in contrast to fast flying bats with short or medium wings of high aspect ratio and rounded wingtips (Norberg & Rayner, 1987). The frequency of wingbeat is negatively correlated with body size and may effectively influence the manoeuvrability and type of prey a bat can handle (Norberg & Norberg, 2012). Further, morphology of the wing and echolocation call design of a bat are related to foraging behaviour, prey availability and the foraging habitat of the bat (Saunders & Barclay, 1992; Bogdanowicz *et al*., 1999).

2.8 Morphology and Diet

Modifications in morphology, particularly in the skull and dental apparatus have been linked to different feeding habits of bats (Freeman, 1981, 1995; Nogueira *et al.*, 2009), which feed on insects, blood, fruits, flowers, amphibians, fish and other small mammals (Hutson *et al.*, 2001). According to Freeman (2000), morphological characteristics including size, length of rostra, thickness of dentary structure, width of face, relative size of molars and characteristics of the stylar shelf are important for predicting the feeding ecology of bats.

The thickness of the dentary structure of insectivores is assumed to be correlated with the toughness of prey (soft or hard-bodied). Thus bats with thick jaws, well-developed cranial crests, high coronoid processes and fewer larger teeth feed primarily on hard-bodied insects such as beetles whereas those with thin jaws, less developed cranial crests, low coronoid processes and more numerous smaller teeth feed on soft-bodied insects such as moths (Freeman, 1979, 1981). For example, morphology rather than echolocation has been suggested as the major determinant of the diet composition in hipposerid and rhinolophid bats (Bogdanowicz *et al.*, 1999). So-called durophagous (“duro” = hard + “phagous” = feeding) and non-durophagous feeding strategies separate both nasal and oral echolocating bats. Nasal emitters have longer faces, enlarged sagittal crests with short muzzle length whereas oral emitters have short, wide faces and robust skulls (Freeman, 2000; Goudy-Trainor & Freeman, 2002).

Feeding mechanisms including bite force, which determines the ability to process food, are important in the partitioning of tropic niches among bat assemblages. Generally, bite force increases with body size in bats, with larger bats using larger bite forces than smaller bats

(Aguirre *et al*., 2002; Santana *et al*., 2010; Senawi *et al*., 2015). Bite force is associated with food hardness in both insectivorous and frugivorous bats. Thus, bats with larger bite force may potentially have increased access to a variety of food resources than those with smaller bite force and may increasingly feed on harder prey (Dumont, 1999; Aguirre *et al*., 2002; Evans & Sanson, 2005). Such relationships may provide possible mechanisms by which bats partition food resources.

2.8.1 Roosting Ecology and Habitat Use of Insectivorous Bats

Roosts are essential resources that offer stable microclimate from adverse weather conditions and protection from predators. They provide sites for social interactions, mating, rearing of young, hibernation and digestion of food (Kunz, 1982; Churchill *et al*., 1997). Several factors affect the choice of roost sites by bats, such as predation risk, energy budget, abundance and proximity to food resources. These factors are largely determined by the interaction between physiological, behavioural and morphological adaptation of bats to their environment. Some bats may use a single roost type such as a cave whereas others may vary roosts seasonally. Further, roost sites close to foraging areas may be used as night roosts to facilitate digestion between feeding bouts, further enhancing social interactions (Kunz, 1982; Hutson *et al*., 2001).

Roost sites used by bats may be natural or artificial. Natural roost sites include caves, cavities in trees and branches, foliage among others, whereas artificial roost sites include crevices in walls of houses, churches, warehouses and abandoned mines. Some bats roost in tents constructed by modifying leaves of some plants, in foliage, cavities in dead and living trees, and others hang freely from trees (Kunz, 1982; Kunz & McCraken, 1996). Bats such as *Lophostoma silvicolum* roost in cavities of active termite nests (Dechmann *et al*., 2004).

Insectivorous bats use different habitat types including both terrestrial and aquatic ecosystems for both foraging and roosting purposes. These habitat types include forest, savanna, woodland, agricultural, urban as well as aquatic habitats such as rivers, lakes and streams (Hutson *et al*., 2001; Russo & Jones, 2003; Avila-Flores & Fenton, 2005). Different habitat mosaics present different feeding opportunities for bats. For instance, depending on the vegetation matrix surrounding an urban area, it may provide a good supply of insects for bats (Rydell, 1992; Russo & Jones, 2003; Avila-Flores & Fenton, 2005). These differential habitat uses in addition to the association of certain landscape features with roosting and feeding areas suggest the need for incorporating land use management plans into bat conservation plans (Rydell, 1992; Hutson *et al*., 2001; Agosta, 2002).

Habitat use by microchiropterans is largely influenced by a complex interaction of morphological, physiological and behavioural adaptations to the environment (Schnitzler & Kalko, 2001; Schnitzler *et al*., 2003). Species with similar morphology are predicted to share similar ecological niches. For instance, echolocation call and morphology of the wing determines how a bat flies and the habitat type in which it forages. Thus, species that feed in open areas use long duration and narrow frequency echolocation calls in comparison to shorter duration and broad frequency echolocation calls used by those in cluttered habitat (Norberg & Rayner, 1987; Schnitzler & Kalko, 2001).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Sites

The study was carried out at eight sites distributed in northern and southern Ghana (Figure 3). Two of the sites were located in the Mole National Park.

3.1.1 Mole National Park

Mole National Park (MNP) (9° 12' - 10° 06' N 1° 25' - 2° 17' W) is the largest wildlife refuge in Ghana covering about 4,840 km². It lies in the Guinea savanna ecological zone and is characterized by a unimodal rainfall pattern and a long dry season. The rainy season starts from April to October and the dry season from November to March. The mean annual rainfall and temperature are about 1,100 mm and 28°C respectively (Ghana Wildlife Division, 2005). Characteristic vegetation types include Sudanian woodland, riparian forest on seasonal rivers, bovals, and rocky outcrops. Bats were captured at identified fly pathways around Brugbani and Gbanwele camps.

3.1.2 Sankana

Sankana is found in the Nadowli-Kaleo district of the Upper West Region. It lies about 19 km from the regional capital, Wa and it is surrounded by rock formations of about 4 km^2. It is found in the Guinea savanna zone and experiences a mean annual rainfall of about 1,100mm. Bats were captured from the network of caves under rock formations around the village.

3.1.3 Sakogu

Sakogu is a community in the East Mamprusi district of the Northern Region of Ghana. It is located at an elevation of about 394 meters above sea level and lies about 2 km from the Gambaga escarpment. The area experiences a single rainfall pattern with an average annual rainfall of between 1000 mm to 1500 mm and a prolonged dry season with an average temperature ranging from of 27.4 °C to 35°C. Bats were captured from under a bridge.

3.1.4 Lilikse

Lilikse is a community in the North-Eastern part of the Upper West Region. It is located in the Sissala East district within the Guinea Savanna vegetation belt. It experiences a single rainy season from April to September and prolonged dry season from early November to March. Bats were captured from caves surrounded by some agricultural landscapes.

3.1.5 Forikrom

Forikrom is located in the Techiman district of the Brong-Ahafo Region of Ghana and lies in the dry semi-deciduous forest zone between the transition zone of high forest in the south and savanna habitats in the north. It experiences an annual rainfall of between 1,250 mm-1,650 mm with the major and minor rainy seasons between April to July and September to October respectively, and the dry season from November to March. Bats were captured at the Boten shrine cave which has a main cavern and satellite caverns serving as roost as well as the surrounding agricultural landscape.

3.1.6 Buoyem

Buoyem is also a community in the Techiman district of the Brong-Ahafo Region with similar climatic conditions to Forikrom. Bats were captured from both Mprisi and Dwamerewa caves which serve as tourist sites. Both caves are separated by a distance of 0.6 km and are located in an agricultural landscape.

3.1.7 Kwamang

Kwamang is found in the Sekyere Central District of the Ashanti Region within the moist semi-deciduous rainforest ecological zone of Ghana. It is characterized by a bimodal rainfall pattern with the major rainy season starting from March to mid-August and the minor season from mid-August to November. The annual rainfall is between 1,200mm –1,500mm with the dry season beginning from December to March. Temperature ranges between 22°C and 30° C with an average temperature of 27°C (Sekyere Central District, 2008). Bats were captured from Ohene Abutia and Mframabuom caves as well as the surrounding agricultural matrix.

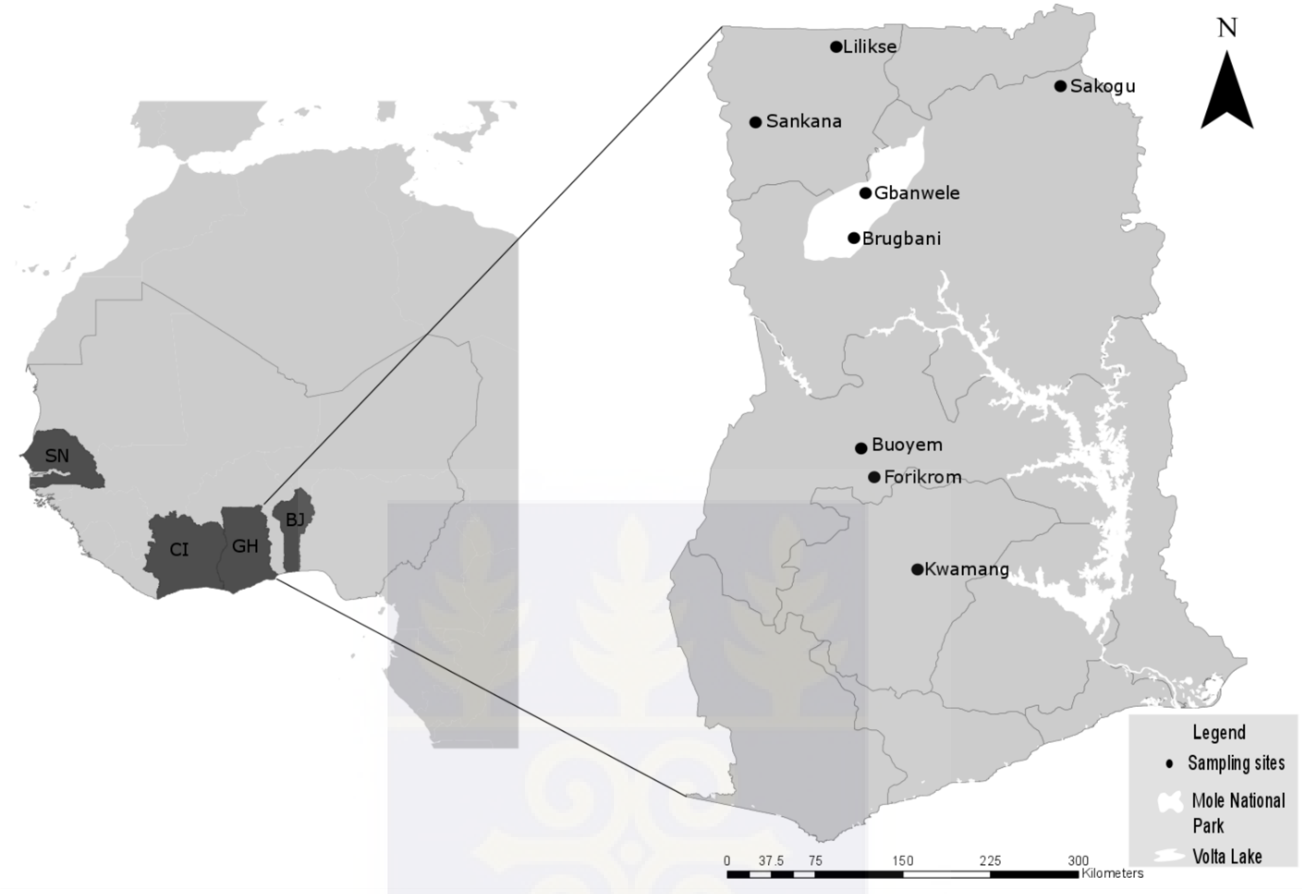

Figure 3: Map of West Africa showing country distribution of bat populations sampled from SN = Senegal, CI = Cote d'Ivoire, GH = Ghana, BJ = Benin and distribution of sampled sites in Ghana.

3.2 Methods

3.2.1 Sampling

Bats were captured with standard and monofilament nylon mist nets (12 x 2.5 m, 3 x 2.5 m and 6 x 2.5 m; mesh size = 16 mm; shelves: 5 Ecotone, Poland) set across the entrances of cave sites as well as identified fly pathways around camp sites, caves and agricultural landscapes of the study areas. Nets were opened at 18:30 hrs and closed at 00:00 hrs GMT. Each cave site and camp site were visited twice per trip from July 2015 to January 2016. Field identification was based on morphological keys according to Rosevear (1965). Forearm length, mass and sex were recorded for all captured bats. Forearm length was measured from the base of the thumb to the end of the ulna with a digital caliper, and mass was measured using a Pesola spring balance (Ecotone, Poland). Sexing of individuals was based on the obvious presence of male and female external genitalia.

3.2.2 Specimen Handling

Twenty-one specimens were collected and preserved in ethanol for further study. Tissue samples were collected from each specimen for molecular studies whereas skulls were extracted from ethanol-preserved specimens for morphological studies. Only adult bat specimens were collected based on the degree of ossification of epyphyseal–diapyseal joints. Voucher specimens and all other biological materials were deposited at the Institute of Evolutionary Ecology and Conservation Genomics, University of Ulm, Germany and the Nation Museum, Prague, Czech Republic.

3.2.3 Molecular Studies

3.2.3.1 Deoxyribonucleic Acid (DNA) Extraction and Purification

Total genomic DNA was extracted from ethanol-preserved tissues using the ZR genomic DNA Tissue Mini Prep kit (Zymo Research, CA, USA) and Geneaid DNA mini kit following the manufacturers' instructions. Tissue samples were digested by adding 95 µl each of double distilled water (ddH_2O) and 2x digestion buffer, 10 µl of proteinase k to 3-5 µg tissue in a microcentrifuge tube, mixed thoroughly by vortexing and incubated at 55°C for 1-3 hours. A volume of 700 µl genomic lysis buffer was added to the tube, mixed thoroughly by vortexing and centrifuged at 10,000 x *g* (13,000 RPM) for one minute to remove insoluble debris. The supernatant was then transferred to a zymo-spin ICC column in a collection tube and centrifuged at 10,000 x *g* for one minute. A volume of 200 µl of DNA pre-wash buffer was added to the spin column in a new collection tube and centrifuged at 10,000 x *g* for one minute, after which 400 µl of g-DNA wash buffer was added to the spin column and centrifuged at 10,000 x *g* for one minute. The spin column was transferred to a clean microcentrifuge tube, 100 µl of DNA elution buffer was added, incubated for 2-5 minutes at room temperature and centrifuged at top speed (14,680 RPM) for one minute to elute the DNA.

The eluted DNA was used in subsequent Polymerase Chain Reactions (PCR). Universal primers L14724 and H15915 (Irwin *et al*., 1991) and specific primer F_1R_1 (modified Sakai *et al*., 2003) were used for both amplification and sequencing of the complete cytochrome-b mitochondrial gene. Total reaction volume for PCR was 25µl with each reaction mixture containing 12.5 µl of Combi PPP master mix, 2.5 µl of DNA extract, 2.0 µl of both forward and reverse primers and 6 µl of ddH_2O. Amplification reactions involved initial denaturation at 94°C for one minute to activate PPPmix, followed by denaturation at 94°C for 40 seconds, 35 cycles of denaturation at 50°C for 40 seconds, annealing for at 72°C for 90 minutes and a final extension for five minutes at 72°C. The amplified products were electrophoresed in TAE buffer using a mini gel system (BIORAD USA) at 100 volts for 30 minutes and gel photographed under ultraviolet (UV) trans-illuminator (Appendix 1).

PCR products with weak amplification or secondary products were subsequently purified directly or from gel using Genome Clean-up Kit (Zymo Research, CA, USA) following the manufacturers' protocol. For direct purification, a volume of 107 µl of DF buffer was added to PCR products, mixed thoroughly by vortexing, transferred to a DF column in a 2 ml collection tube and centrifuged for one minute at 14-16000 x *g*. The flow-through was discarded, 600 µl of wash buffer was added to the DF column and allowed to stand for one minute and centrifuged for one minute at 14-16000 x *g*. The flow-through was discarded and the DF column in a collection tube was centrifuged 14-16000 x *g* for three minutes to dry the column matrix. The dry DF column was transferred into a new 1.5 ml microcentrifuge tube, 20 µl of elution buffer was added to the centre of the column matrix and allowed to stand for two minutes to ensure that the elution buffer was completely absorbed. Purified DNA was obtained by centrifuging for 14-16000 x *g* for two minutes.

For purification of PCR products from gel, portions of agarose gel slice weighing about 300 mg, and containing relevant DNA fragments were cut off, transferred into a 1.5 ml microcentrifuge tube and 500 µl of DF buffer was added and mixed thoroughly by vortexing. Gel slice was dissolved by incubating at 55°C for 10-15 minutes and later cooled at room temperature, after which 800 µl of sampled mixture was transferred into a DF column in a 2 ml collection tube and centrifuged at 14-16,000 x *g* for one minute. The flow-through was then discarded, and 400 µl of W1 buffer was added to the DF column in a collection tube and centrifuged at 14-16,000 x *g* for one minute. The flow-through was discarded, 600 µl of wash buffer was added and made to stand for one minute. The sampled mixture was centrifuged at 14-16,000 x *g* for one minute and the flow-through was discarded and centrifuged again at 14-16,000 x *g* for three minutes to dry the column matrix. The dried DF column was transferred into a new 1.5 ml microcentrifuge tube, 20 µl of elution buffer was added to the centre of the column matrix and allowed to stand for two minutes to ensure that the elution buffer was completely absorbed. Purified DNA was obtained by centrifuging at 14-16,000 x *g* for two minutes. All laboratory work was carried out the Institute of Evolutionary Ecology and Conservation Genomics, University of Ulm, Germany under the supervision of Dr. Peter Vallo.

3.2.3.2 Sequence Alignment and Analysis

Sequences of amplified PCR products were obtained from a commercial biotechnology company (Macrogen, Amsterdam, Netherlands) using the Big-Dye Terminator sequencing chemistry (Applied Biosystems, Foster City, CA, USA) on ABI 3730xl sequencer. Sequence reaction contained 0.5 µl each of purified DNA and the same primers. Generated sequences were assembled and edited manually in Sequencher v. 4.7 (Gene code, Ann Arbor, MI, USA) and alignments were created in BioEdit v.7.0.1 (Hall, 1999). Polymorphism, haplotype diversity (*h*) and nucleotide diversity (π) of the cty b sequences were assessed using DNASP 5.10 (Librado & Rozas, 2009). Sequences were then reduced to haplotypes to facilitate reconstruction of a phylogenetic tree. A median-joining network of haplotypes was constructed in program

POPART (http://popart.otago.ac.nz). Additional sequences of West African *H.* aff. *ruber* were downloaded from the GenBank for comparative analysis (Appendix 2). *Hipposideros gigas* and *Hipposideros abae* were used as an outgroup in the phylogenetic analysis.

Reconstruction of phylogenetic relationships of haplotypes was inferred under maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods (BA). Phylogenetic relationships under MP and ML were carried out using heuristic searches with tree bisection-reconnection (TBR) swapping algorithm on 100 random sequence in the software PAUP* 4.10b (Sinauer Associates, Sunderland, MA, USA) and PhyML 3.0 (Guindon *et al.*, 2010), respectively. The consistency of branching patterns of trees was then assessed using bootstrap analysis with 1,000 replicates for both MP and ML (Felsenstein, 1985). The best-fit nucleotide substitution model for the set of aligned sequences was determined using the Akaike information criterion (AIC) in the software modelTest 3.7 (Posada & Crandal, 1998). AIC represents a balance between complexity of the model and goodness-of-fit of the model. The Hasegawa, Kishino and Yano model with gamma distribution among-site rate variation (GTR + I + Γ) was selected as the most appropriate for sequence evolution.

Bayesian analysis was carried using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). It is computationally more efficient than the bootstrapping maximum likelihood method (Larget & Simon, 1999). Two independent simultaneous Metropolis-coupled Markov chain Monte Carlo (MCMC) simulation runs for 1,000,000 generations, sampled at every 100th generation beginning with random trees was used. A final consensus tree was generated using a 50% majority rule consensus tree after an initial ‘burn in’ of 2,500 trees. Genetic divergences were calculated as pairwise Kimura two-parameter genetic distances (K2P)(Kimura, 1980), which are comparable to other published work (e.g. Vallo *et al*., 2008, 2011).

3.2.4 Morphological Studies

3.2.4.1 Source of Specimens

A total of 67 *H.* aff. *ruber* skulls from Ghana and Senegal was used for geometric morphometric analysis (Table 1). All samples were obtained from the Institute of Evolutionary Ecology and Conservation Genomics, University of Ulm, Germany and the Nation Museum, Prague, Czech Republic.

Table 1: Number of specimen included in geometric analysis according to cranium view, gender, lineages, country and locality from Ghana.

View	Gender		Lineages			Country		Ghana	
	Female	Male	D1	D2	D3	Ghana	Senegal	Northern	Southern
Dorsal	31	34	12	16	20	55	12	35	20
Ventral	27	35	12	17	18	55	11	36	19
Lateral	26	36	14	16	18	52	13	33	19

3.2.4.2 Geometric Morphometrics

All specimens were consistently oriented and photographed using a Nikon D100 digital camera with a 3,000 x 2,000-pixel resolution. All skulls were mounted on a plasticine material with graph paper, photographed at same distance and focus by fixing the camera to a laboratory camera stand so as to minimize distortions and bias resulting from photographing. The software TpsUtil 1.68 (Rohlf, 2015) was used to create an empty TPS file from the directory with image files. Two-dimensional coordinates of 10, 8 and 13 landmarks corresponding to dorsal, ventral and lateral views, respectively, were obtained using the digitization software TpsDig 2.25 (Rohlf, 2015). Type II landmarks (Bookstein, 1991) and semi-landmarks were used in the digitization of images and all landmarks were digitized on the left half of the skull to avoid the effect of bilateral symmetry. Semi-landmarks were appended to landmarks using the software TpsUtil 1.61 (Rohlf, 2015) and equally treated as other landmarks in all statistical analyses (Gunz & Mitteroecker, 2013).

3.2.4.2 Definition of Landmarks

- Dorsal view (Plate 2A): (1) Midpoint of posterior curvature of inter-nasal openings, (2) exoccipital, (3) most posterior internal part of the zygomatic arch, (4) maximum curvature of the anterior margin of the zygomatic arch, (5) most anterior internal point of the zygomatic arch, (6) most anterior point of the premaxillae, (7-10) curvature of the nasal region.
- Ventral view (Plate 2 B): (1) Midpoint of the posterior curvature of the inter-nasal opening, (2) posterior limit of the palatine, (3) anterior limit of the foramen magnum, (4) posterior limit of the foramen magnum, (5) lateral limit of the foramen magnum (right), (6) most posterior internal point of the zygomatic arch, (7) maximum curvature of the anterior margin of the zygomatic arch, (8) most anterior internal part of the zygomatic arch.

- Lateral view (Plate 2. C): (1) Exoccipital, (2) ventral point of occipital condyle, (3) extreme ventral point of supraoccipital (4) anterior limit of mandibular condyle, (5) Posterior limit of mandibular condyle, (6) 3rd molar post extremity, (7) alveolar limit between 2nd premolar and 1st molar, (8) alveolar limit between 1st and 2nd premolar, (9) most anterior point of the premaxilla, (10-13) curvature of the nasal region.

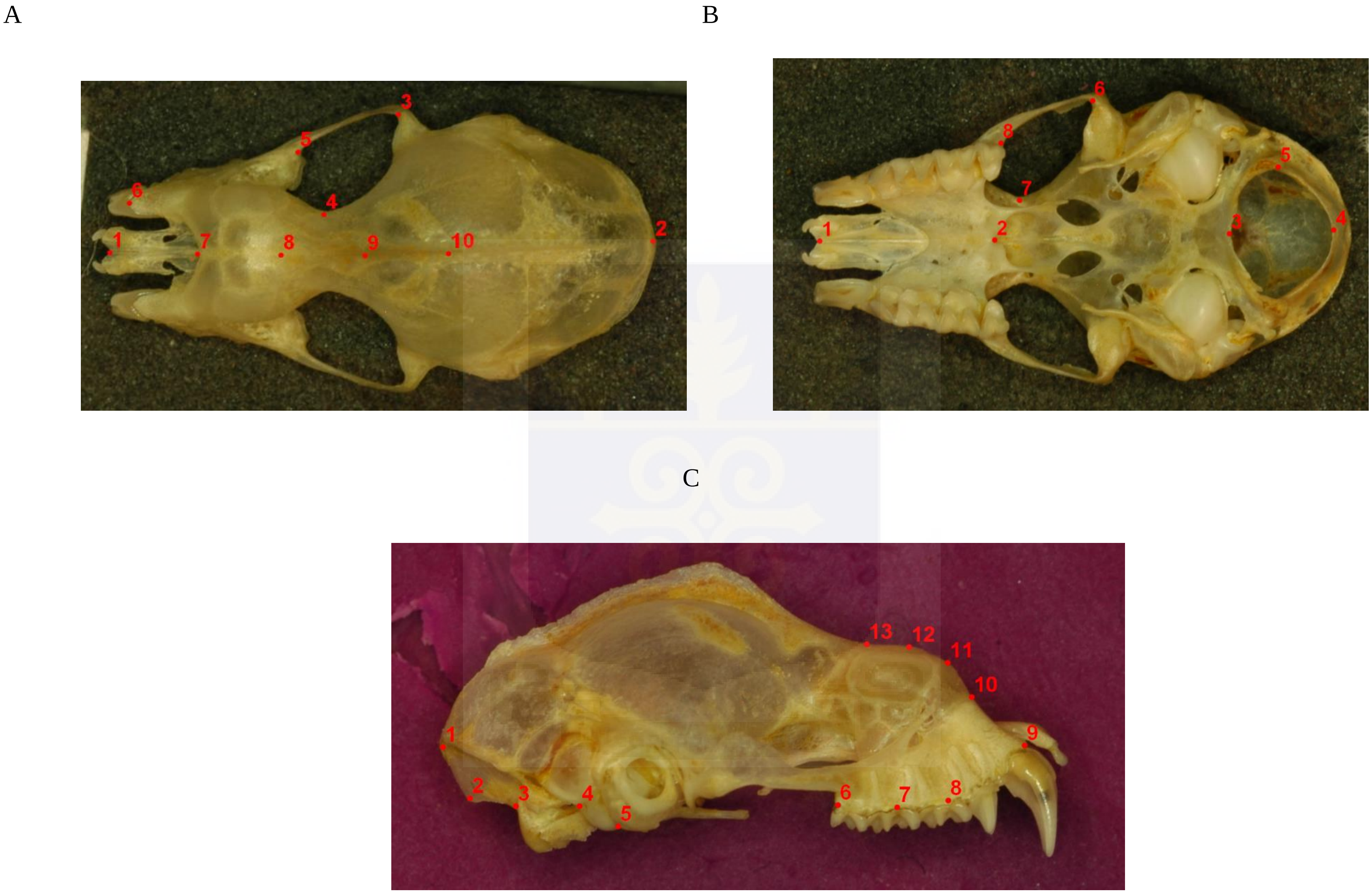

Plate 2: Landmarks on the Dorsal (A) Ventral (B) and lateral (C) cranium views used in this study.

3.2.4.3 Morphological Analysis

Morphological variations in the skulls were studied by separately analyzing size and shape components. Landmark configurations of all specimens were superimposed to obtain mean configuration using Generalized Procrustes Analysis (GPA). This allowed for shape and size separation and projection of shape coordinates into a Euclidean space to allow for further statistical analysis (Rohlf & Slice, 1990; Bookstein, 1991). GPA analysis produces the best fit of landmark configurations according to a least-squares criterion by removing non-shape variations resulting from scaling, rotation and translation (Rohlf & Slice, 1990).

Shape data was inspected for outliers based on a model that assumes that the data are multivariate normally distributed in the program MorphoJ. A covariance matrix of the GPA shape coordinates was generated and used for initial Principal Component Analysis of shape coordinates. Shape differences among lineages were assessed using Canonical Variates Analysis (CVA) with 10,000 permutations. Discriminant Function Analysis (DFA) with leave-one-out cross validation was used to assess the strength of classification among groups. Further, variation in mean shape was tested with permutation test with 1,000 iterations using Mahalonobis distance (D) and the Procrustes distance (d). The Mahalanobis distance shows how unusual an individual's shape is relative to others in the population whiles the Procrustes distance is a measure of absolute magnitude in shape deviation (Klingenberg & Monteiro, 2005). Variations in shape were visualized in the form of wireframe graphs (landmarks connected with straight lines). All shape analyses were done using the software MorphoJ v 1.06d (Klingenberg, 2011).

Size was estimated as the centroid size; the square root of the sum of the squared distances of all landmarks and the objects centroid. It represents a good measure of the size variable since it does not induce a correlation between shape and size (Bookstein, 1991). Differences in log centroid size of lineages were tested with Analysis of Variance (ANOVA) and Tukey's Honest Significant Difference (HSD). Welch's t-test was used to assess log centroid size differences of geographical locations and among sexes. Differences were depicted with boxplots and all size analyses were done in R 3.2.2 (R Core Team 2015).

3.3 Ethical Considerations

Capturing and handling of bats were in accordance with Ghanaian and international legislation. Permits for capture, handling, capturing and processing of all animals were obtained from the Wildlife Division of the Forestry Commission of Ghana. Exports of bat samples were conducted under a state agreement between the Republic of Ghana and the Federal Republic of Germany.

CHAPTER FOUR

RESULTS

4.1 Genetic diversity of *Hipposideros* aff. *ruber*

Analyses of the 41 cytochrome (cyt) *b* mitochondrial gene [725 base pairs (bp)] sequences of the Ghanaian population of *Hipposideros* aff. *ruber* (D lineage) revealed 62 polymorphic sites made of 57 parsimony informative sites and five singleton variable sites corresponding to 19 unique haplotypes. Specimens from Sakogu and Lilikse sites from northern Ghana contributed five haplotypes each while Gbanwele and Forikrom contributed only one haplotype each.

Three distinct genetic lineages were recovered based on the median-joining tree of the haplotypes from Ghana and are referred to as D1, D2 and D3 in accordance with the original D lineage (Vallo *et al.*, 2008; 2011) (Figure 4). The population was separated into northern (D2, D1) and southern Ghana populations (D3). Kimura-2-parametric (K2P) genetic distance within the sub-lineages ranged from 0.0-1.5%. Interestingly, divergence of up to 6.1% was observed between haplotypes from southern (Buoyem and Kwamang) and northern (Lilikse and Sakogu) Ghana sites attributable to D3 and D2 respectively (Table 2).

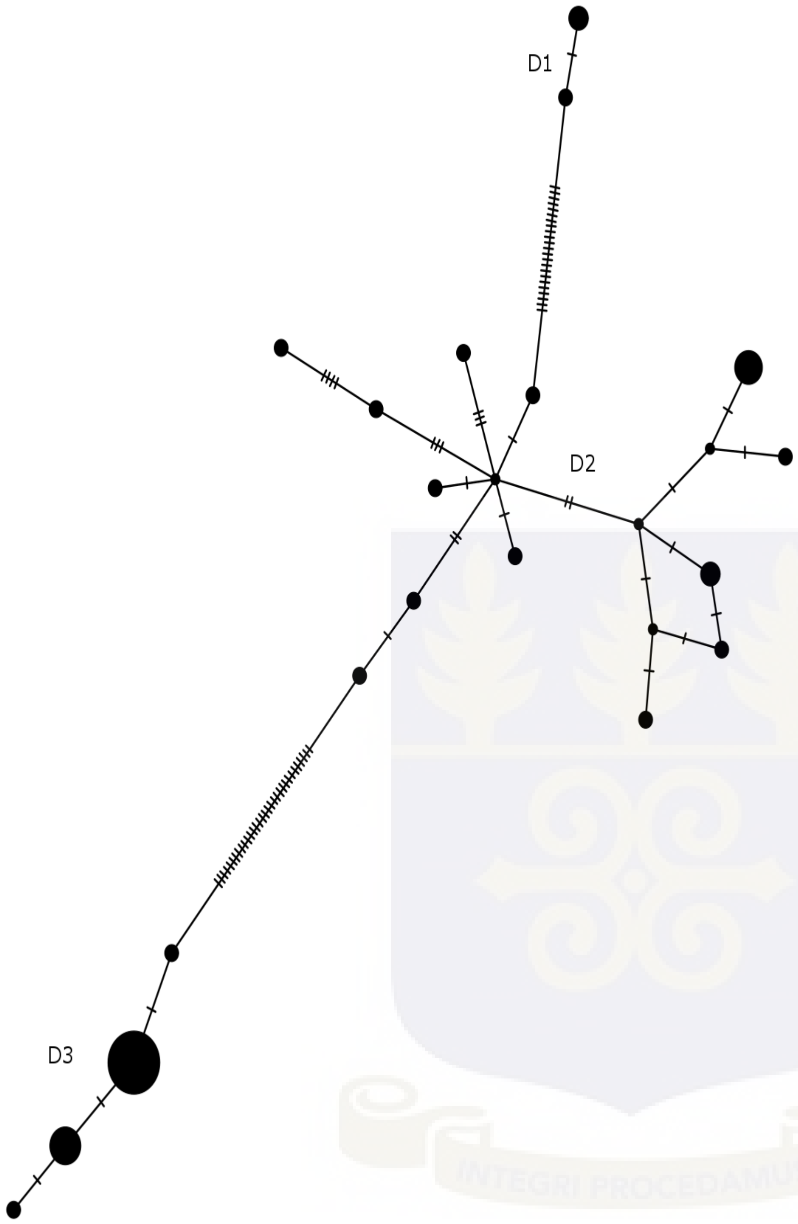

Figure 4: Median-joining network of *Hipposideros* aff. *ruber* haplotypes from Ghana. Size of circle is proportional to haplotype frequency. Mutations are shown as hatch mark.

4.2 Phylogenetic Reconstruction of *Hipposideros* aff. *ruber*

Evolutionary relationships among Ghanaian populations of *H.* aff. *ruber* and other West African forms were analyzed using 63 cyt *b* sequences (725 bp); from Ghana (41), Senegal (19), Cote d'Ivoire (2) and Benin (1) (Appendix 2). Eighty-one polymorphic sites comprising of 71 parsimony informative sites and 10 singleton variable sites were recovered. There were 35 unique haplotypes (h =0.9411, π = 0.03268) of which nine were shared among a total of 36 individuals (Appendix 2). There were no shared haplotypes between the samples from the different countries. Sub-lineages D1 and D2 consisted of 13 and 17 haplotypes each from 19 and 21 specimens, respectively, whereas D3 was formed by six haplotypes from 23 specimens.

Phylogenetic reconstruction using maximum parsimony (MP) and maximum likelihood (ML) methods resulted in trees with similar topology to the Bayesian consensus tree. Three lineages were recovered using all methods. Haplotypes from southern Ghana (Kwamang, Forikrom, Buoyem) form a monophyletic group with specimens from Cote d'Ivoire in D3 with high posterior probability and bootstrap support. Within lineage D1, two haplotypes from northern Ghana (Sakogu and Lilikse) are in sister relationship within a larger Senegalese clade. Senegalese haplotypes cluster as sisters at the top of the lineage D2 in an unresolved relationship to haplotypes from Ghana and Benin. Remaining specimens from Sakogu and Lilikse from Ghana form a well-supported monophyletic group within D2 (Figure 5).

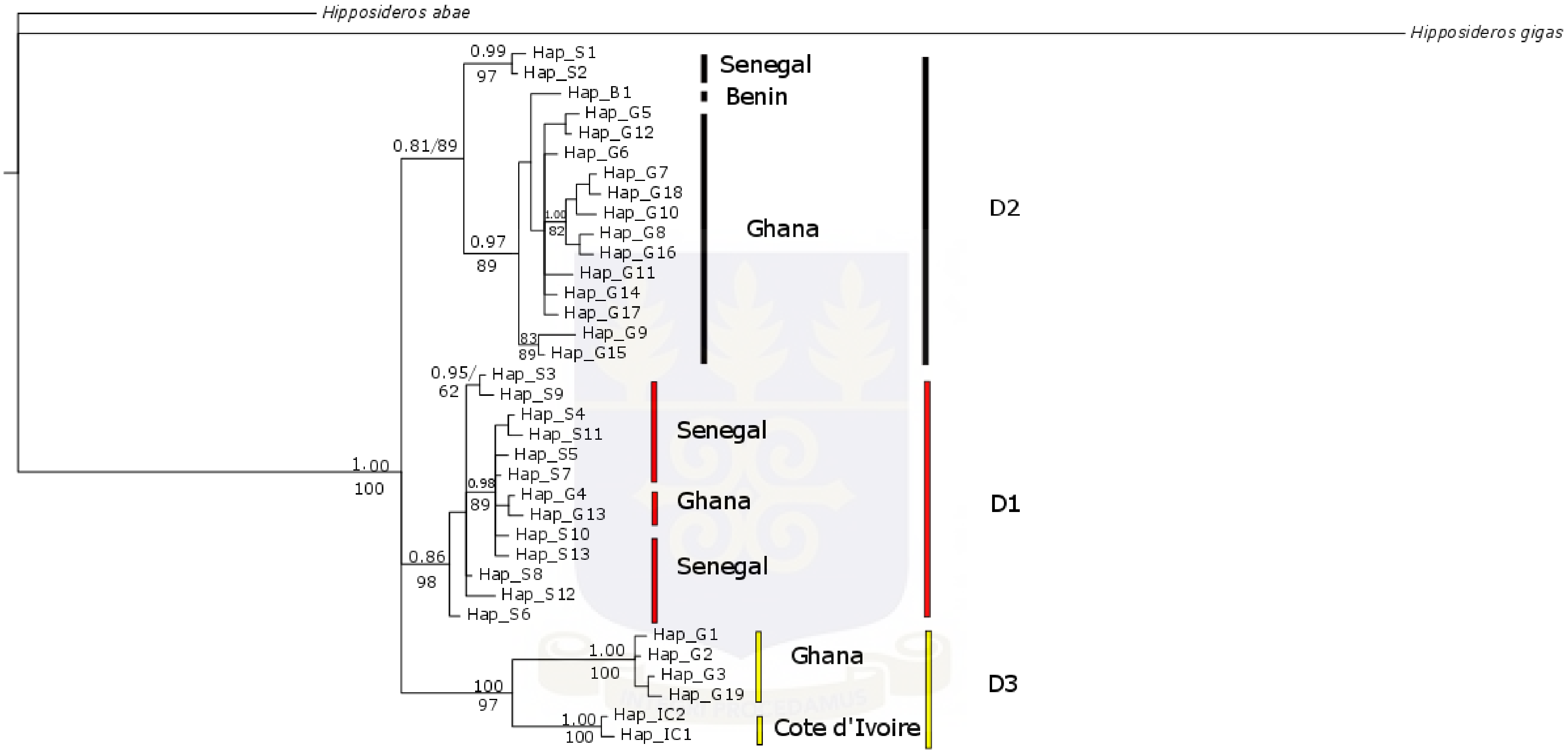

Figure 5: Bayesian 50%-majority consensus tree with support for major nodes. Posterior probability of Bayesian analysis indicated above nodes and bootstrap support for MP below branches. Color scheme is consistent with the Figure 7.

K2P genetic divergence within group D3 was 0.1-3.7% for samples originating from Ghana and Cote d'Ivoire whereas samples from Ghana and Senegal grouping in D2 differed by 0.1-2.5%. Within group D1, genetic variation ranged from 0.1-1.1% for samples from Ghana, Senegal and Benin. Sequence divergence between the sub-lineages ranged from 0.1-6.1 (Table 2).

Table 2: Pairwise genetic divergences among phylogenetic lineages of West African *Hipposideros* aff. *ruber* [K2P- Kimura two-parameter distance (%)].

K2P%	Ghana D3	Ghana D2	Ghana D1	Senegal D1	Senegal D2	Cote d'Ivoire D3
Ghana D2	4.6-6.1					
Ghana D1	5.1-5.7	3.3-4.2				
Senegal D1	4.5-5.7	2.7-4.2	0.1-1.1			
Senegal D2	4.3-4.9	1.8-2.5	3.6-3.7	2.7-3.9		
Cote d'Ivoire D3	3.3-3.7	4.8-5.7	4.2-4.5	3.9-4.5	4.9-5.2	
Benin D2	5.4-5.8	0.7-1.1	3.7-3.9	3.3-3.9	2.1-2.3	4.9-5.1

4.3 Morphological Variation

4.3.1 Size

Analyses of the natural logarithm of centroid size for *H. aff. ruber* populations from Ghana and Senegal showed significant differences for lateral (t = 4.97, d.f. = 19.07, P = 0.00008), ventral (t = 4.44, d.f. = 13.68, P = 0.0005) and dorsal views (t = 4.82, d.f. = 17.77, P = 0.0001; Appendix 3a). Mean differences in centroid sizes also differed significantly for populations from northern and southern Ghana for all three views analyzed (Table 3).

Table 3: Welch t-test results for geographical variation of *Hipposideros* aff. *ruber* populations from northern and southern Ghana. (t = t value, d.f. = degree of freedom, *P*-value = significance level).

View	Northern Mean	Southern Mean	t	d.f.	*P*-value
Lateral	7.194	7.179	2.706	44.899	0.00959*
Ventral	6.847	6.829	4.074	45.941	0.00018*
Dorsal	6.881	6.861	4.548	47.938	0.00003*

Among all three lineages, mean centroid size for the dorsal (F =11.47; d.f. = 2, 45; P = 0.00009), ventral (F =9.63; d.f. = 2, 44; P = 0.00034) and lateral views (F = 7.96; d.f. = 2, 45; P = 0.00109) differed significantly. The greatest size differences were observed among individuals of D2 in all three views analyzed (Appendix 3b). Average centroid size of lineage D2 differed significantly from D1 for all views and D3 for both ventral and dorsal views after the Tukey's HSD post-hoc test ($P < 0.05$).

Mean centroid size for males was significantly different from those of females for both lateral and ventral views (Table 4). Among the northern and southern Ghana populations, significant size differences among sexes were recorded for lateral ($F = 2.975$; d.f. = 3,45; $P = 0.0415$), ventral ($F = 5.942$; d.f. = 3,47; $P = 0.0016$) and dorsal views ($F = 13.48$; d.f. = 3, 49; $P < 0.0001$). According to the Tukey's HSD test, males belonging to the northern population were significantly larger in size than males and females from the south for both ventral and dorsal views but larger than females for the lateral view. Also, males of the north were larger than females of the north for only the dorsal view.

Table 4: Welch t-test of log centroid size for sexes in all three views (n = sample size, t = t value, d.f. = degree of freedom, ♂ = males, ♀ = females).

View	**n**	**♂ Mean**	**n**	**♀ Mean**	**t**	**d.f.**	***P-value***
Lateral	36	7.189	26	7.170	3.111	51.84	0.0030*
Ventral	35	6.840	27	6.829	1.874	53.25	0.066
Dorsal	34	6.877	31	6.859	3.498	62.612	0.0008*

*P < 0.05

4.3.3 Shape

Principal component analysis (PCA) of the shape component showed no separation for *H.* aff. *ruber* populations from both Senegal and Ghana. Shape analyses were therefore restricted to populations from northern and southern Ghana, among lineages and sexes. A total of 16, 22 and 12 principal component (PC) axes respectively were generated for dorsal, lateral and ventral views of the Ghanaian population. Ninety-nine percent of the overall shape variance was explained as 12 by PC axes for dorsal, 17 for lateral and 11 for ventral view. The first two PC accounted for 43.6% and 19.6% of shape changes for the dorsal, 26.7% and 13.4% for the ventral and 29.2% and 15.6% for the lateral view (Figure 6). The Mahalanobis (D) and Procrustes distances (d) between mean shape of northern and southern populations were significant at $P < 0001$ for dorsal ($D = 3.0725$, $d = 0.0212$), lateral ($D = 3.8715$, $d = 0.0107$) and ventral ($D = 2.2298$, $d = 0.0137$) views. Leave-One-Out cross validation correctly assigned 81.8% and 78.9% for northern and southern populations, respectively, for the lateral, 80.0% and 85.0% for the dorsal and 80.5% and 68.4% for the ventral view.

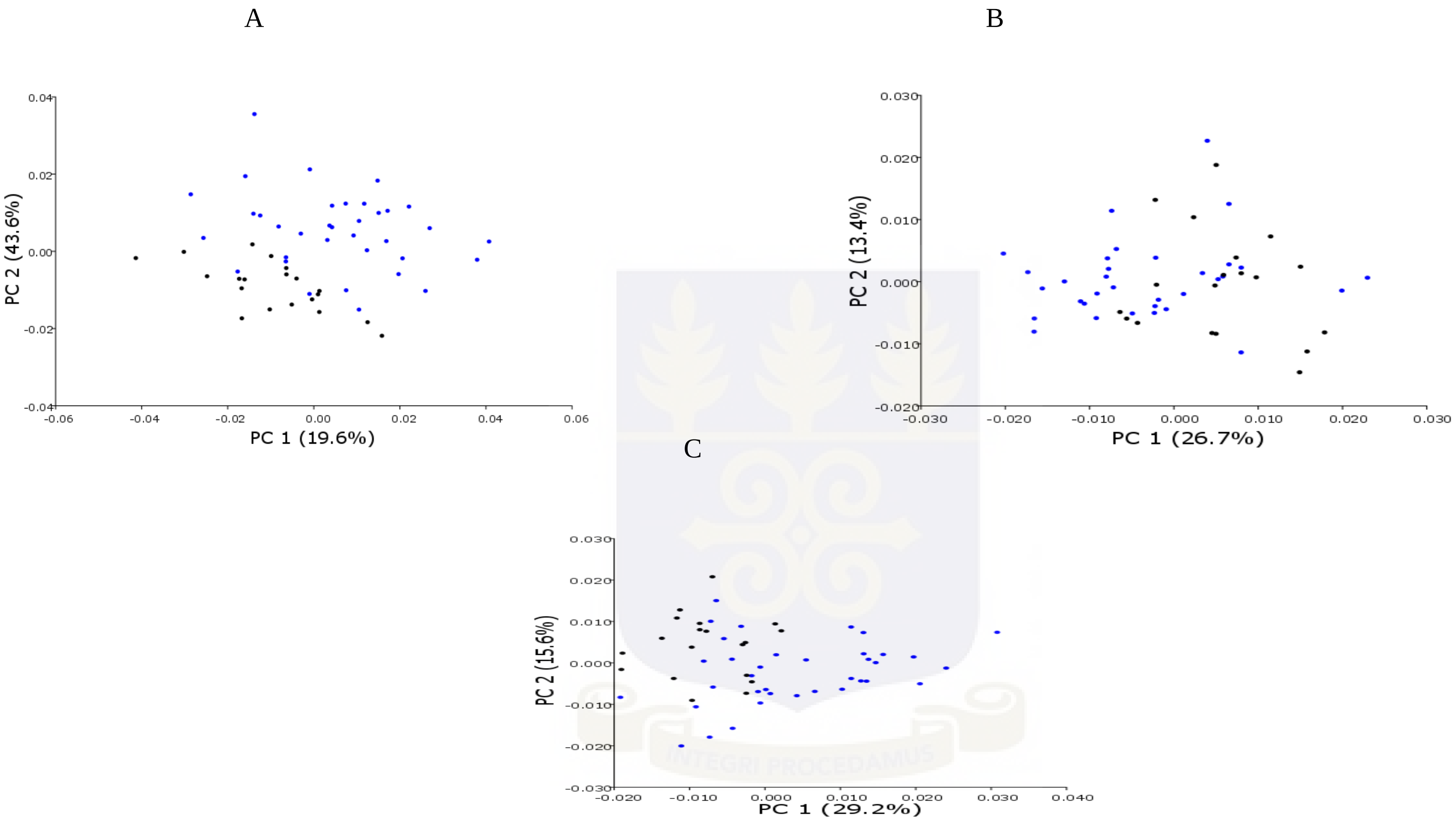

Figure 6: First principal component versus second principal component for the dorsal (A), lateral (B) and ventral (C) skull views of northern (blue circles) and southern (Black circles) Ghana populations of *Hipposideros* aff. *ruber*. Numbers in parenthesis are percentage shape changes for a given axis.

The first two axes of Canonical Variate Analysis (CVA) explained 89.04% and 10.96% of the shape variation for the dorsal, 65.41% and 34.59% for ventral, and 80.01% and 19.01% for the lateral side among the three lineages. The first axes of canonical variate (CV1) accounted for most of the variation and clearly separated group D3 in both dorsal and lateral views. Groups D1 and D2 slightly overlapped and were considerably shifted towards the negative values on CV1 in comparison to D3 except for the ventral view (Figure 7).

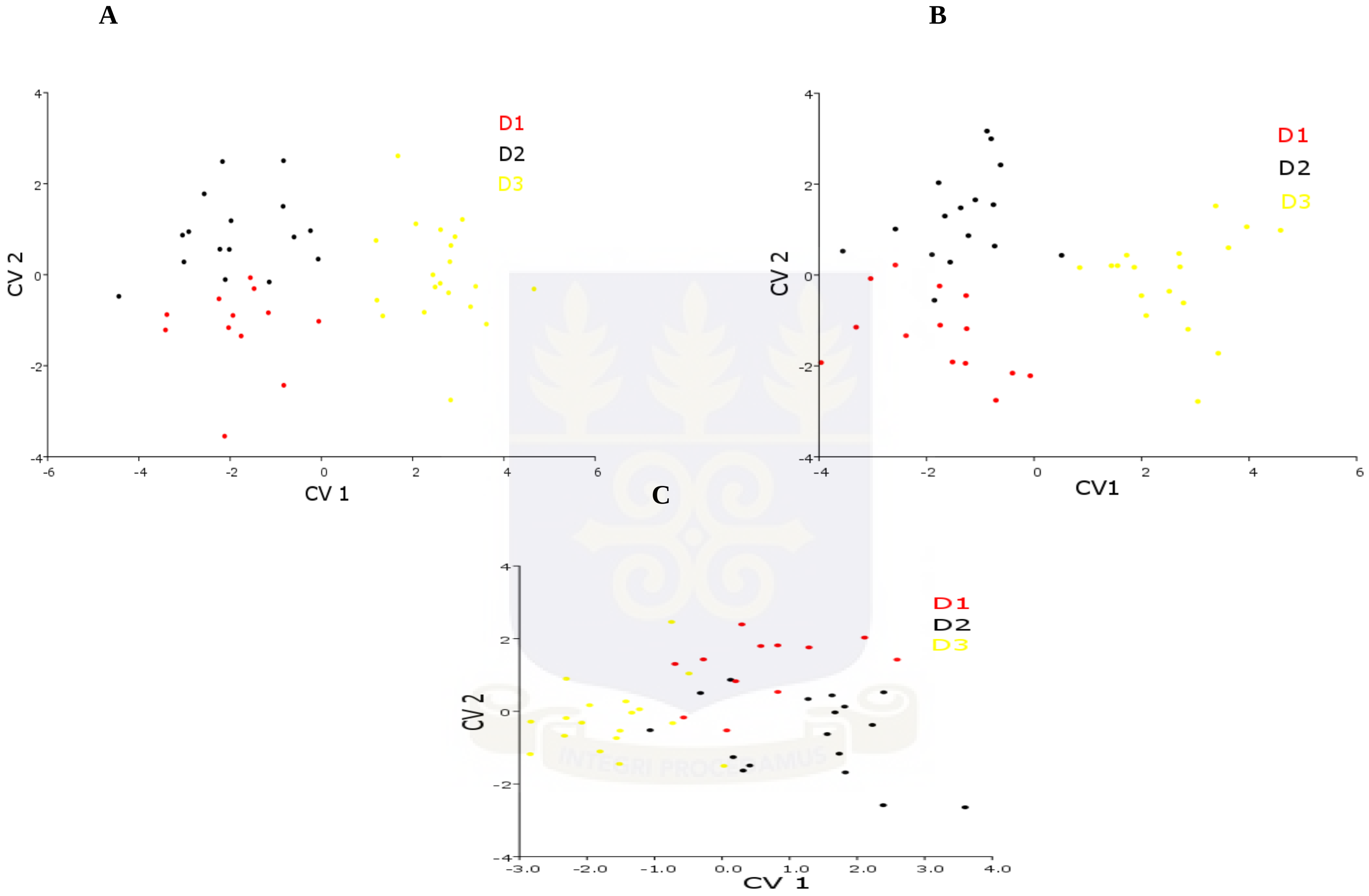

Figure 7: First canonical variate versus second canonical variate of dorsal (A), lateral (B) and ventral (C) cranium views for the mitochondrial D sub-lineages of *Hipposideros* aff. *ruber*

Discriminate Function Analysis (DFA) showed relatively high scores for all sub-lineages across all skull regions analyzed. It correctly assigned all specimens 100% for the lateral view but Leave-One-Out cross validation correctly assigned 50% and 68.7% for groups D1-D2, 71.4% and 83.3% for D1-D3, 81.2% and 83.3% for D2-D3 while returning 66.6% and 62.5% for D1-D2, 91.6% and 90.0% for D1-D3 and 75.0% and 85.0% for D2-D3 for the dorsal view. Regarding the ventral view, 66.6% and 47.0% for D1-D2, 75.0% and 72.2% for D1-D3 as well as 70.5% and 83.3% for D2-D3 were correctly assigned. Significant Mahalanobis and Procrustes distances were recorded for the mean shape of groups D1-D3 and D2-D3 for all three views (Table 5).

Table 5: Classification results based on Mahalanobis and Procrustes distances between mean shapes of West African *Hipposideros* aff. *ruber* lineages.

Skull region	**Groups**	**Mahalanobis Distance (D)**	***P*-value**	**Procrustes Distance (d)**	***P*-value**
Lateral	D1-D2	4.2587	0.2760	0.0089	0.1120
	D1-D3	8.3806	0.0030*	0.0123	< .0001*
	D2-D3	7.8307	<. 0001*	0.0144	0.0010*
Ventral	D1-D2	2.0228	0.2330	0.0100	0.0670
	D1-D3	3.1303	0.0100*	0.0145	0.0010*
	D2-D3	3.0251	0.0030*	0.0150	< . 0001*
Dorsal	D1-D2	3.1499	0.1530	0.0082	0.4660
	D1-D3	6.5949	< . 0001*	0.0221	< . 0001*

	D2-D3	4.7753	< . 0001*	0.0204	< . 0001*

P < 0.05

For the pooled data on sexes, the Mahalanobis and Procrustes distances between mean shape were not significant for the ventral view (D = 1.0891, *P* = 0.2840; d = 0.0062, *P* = 0.1970) with Leave-One-Out cross validation correctly classifying 13 out of 27 females and 17 out of 35 males whereas 23 out of 36 males and 12 out of 26 females were classified correctly for lateral view (D =1.6124, *P* = 0.3390; d = 0.0047, *P* = 0.5530). In the dorsal region, 20 out of 31 females were classified correctly as compared to 21 out of 34 males (D = 1.5944, *P* = 0.0350; d = 0.0011, *P* = 0.007).

Analysis of shape differences among sexes of northern and southern populations of Ghana revealed similar results of non-significant Mahalanobis and Procrustes distances for all three cranial views. However, cross validation correctly assigned seven out of 12 females and 10 out of 19 males from the north, whiles four out of five females and 10 out 13 males were correctly assigned for the southern population based on the lateral view. Similarly, seven out of 16 females and 12 out of 17 males for the northern population and four out of seven females and six out of 13 females were correctly assigned based on the dorsal view. For the ventral view, cross validation correctly assigned 11 out of 15 females and 13 out of 18 males for the north while correctly classifying two out of five females and seven out of 13 males correctly.

The most distinguishing shape differences were observed in the region of the zygomatic arch for both northern and southern populations of Ghana as well as among sub-lineages. Based on the dorsal and ventral views, southern populations and sub-lineage D3 have a slightly wider zygomatic arch in comparison to northern populations and sub-lineage D1 and D2, respectively (Figure 8). D1 and D2 are not distinguishable based on the shape for all three views analyzed. There were also slight shape changes in the area of the nasal inflation based on the lateral view. Lineage D3 and the southern population show a slightly abrupt nasal inflation and a high position of the most anterior point of the premaxilla in comparison to D1 and D2.

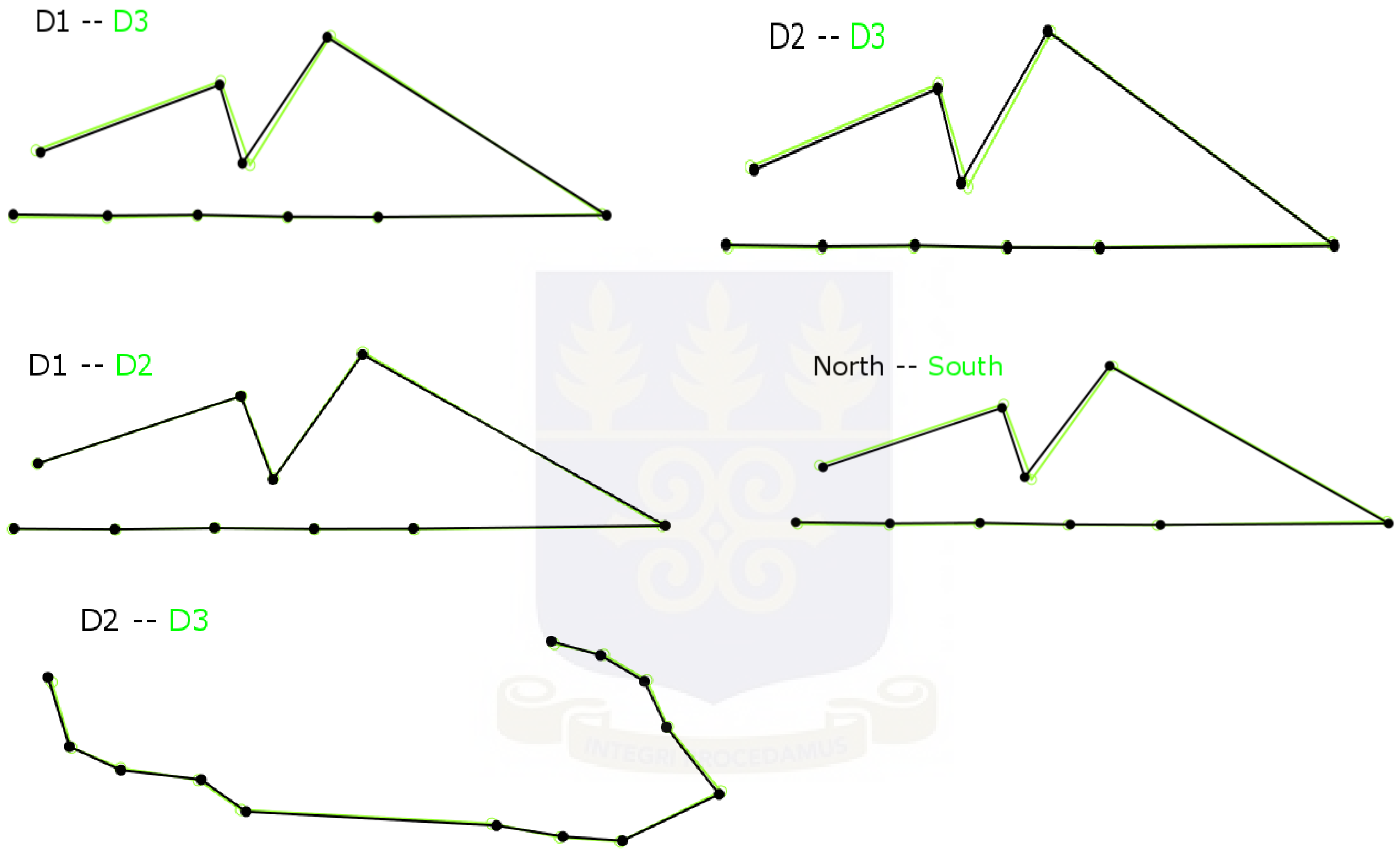

Figure 8: Shape comparison of the mitochondrial D sub-lineages and northern and southern Ghana population of *Hipposideros* aff. *ruber* based on dorsal and lateral cranium views.

CHAPTER FIVE

DISCUSSION

5.1 Genetic Diversity of *Hipposideros* aff. *ruber*

Cryptic diversity of Noack's leaf-nosed bat (*Hipposideros* aff. *ruber)* was investigated using both molecular and morphological data. *Cytochrome b* sequence analysis of West African *H.* aff. *ruber* revealed three deeply divergent lineages with K2P genetic distance of 0.1-6.1%. Populations from Ghana showed diverse haplotypes suggesting higher diversity in this region or possibly an indication of adequate sampling from different vegetation zones relative to the other West African countries. Absence of haplotypes from the Cote d'Ivoire and Benin from the other lineages may be due to low sample size or perhaps an attribute of habitat selection.

Diverse intra-generic and intra-specific relationships have been documented for bats in general and the family Hipposideridae. The presence of three deeply divergent lineages within the so-called D lineage confirms the findings of deep intra-lineage divergences within the *H.* aff. *ruber* complex (Vallo *et al*., 2011; 2008). According to Bradly and Baker (2001), genetic divergence < 2% is characteristic of within-species variation. Intra-specific divergence values ranging from 0.0-5.9% have been documented for bats (Baker & Bradley, 2006), 2.4–3.6% for *H.* aff. *ruber* from Senegal (Vallo *et al*., 2011) and 1.2–2.1% for D lineage from Ghana (Baldwin, 2015). Furthermore, genetic distance of 3.9–4.1% has been documented for cryptic Asian species *H. khakhouayensis* and *H. rotalis* (Guillén-Servent & Francis, 2006) and 6.5-6.8% among 142 kHz *H. bicolor* and 131kHz *H. bicolor* (Kingston *et al*., 2001). Hybridization resulting from past introgression of mitochondrial DNA has also been inferred to explain low sequence divergence among different genera of Central African fruit bats, *Epomophorus gambianus* and *Micropteropus pusillus* with sequence divergence of up to 2.5% (Nesi *et al*., 2011) as well as between West African small-sized house bats, *Scotophilus leucogaster* and *Scotophilus* aff. *nigritellus* with divergence of 2.6–2.8% (Vallo *et al*., 2013).

The observed genetic divergence of ≥ 6.1% between the southern (Buoyem and Kwamang) and northern populations (Lilikse and Sakogu) assigned to sub-lineages D3 and D2, respectively, could be considered as separate species based exclusively on known limits of sequence divergence between cryptic species (Bradley & Baker, 2001; Baker & Bradley, 2006). According to the genetic species concept of Baker and Bradley (2006), percentage genetic distance greater than 5% represents a reliable initial indicator for recognition of distinct species. Even so, relatively deep divergence among lineages does not necessarily dictate species status since substantial nuclear gene flow may occur among such groups due to matrilineal inheritance of the mitochondrion gene (Avise, 2004). Among Chiropterans, substantial gene flow has been documented for two previously-predicted separate species for the North American bats *Myotis lucifugus lucifugus* and *Myotis lucifugus carissima* with sequence divergence of up to 5% (Lausen *et al.*, 2008). Thus, it is necessary to document reproductive isolation of the two sub-lineages in Ghana to prove their potential species status.

Relationships between genetic divergence and geographical locations among studied populations suggest a complex relationship. Geographically close populations (ca.660 km) from Ghana and Cote d'Ivoire differ genetically by 3.7% as compared to 2.5% for a more distantly-separated population from Senegal and Benin (ca. 2000 km). Similar patterns have been documented for the *H*. aff. *ruber* complex from other West African sites. Vallo *et al*. (2011) reported similar relationships between geographically-close Ghana and Benin populations with genetic divergences of over 5% and 2% for geographically distant Senegal and Benin populations. Sequence divergence of up to 2.9% has been documented for populations from Liberia, Cote d'Iviore and Ghana while divergence over 5% has been recorded for populations from Senegal, Liberia and Cote d'Iviore (Monadjem *et al*., 2013). Mechanisms of isolation by distance has been used to explain such patterns given that these populations are allopatric (Vallo *et al*., 2011). In Ghana, genetically distant populations pertaining to the D lineage are clearly separated by distance even at relatively short geographical distances of less than 350 km (Baldwin, 2015).

Climatic fluctuations of the late Miocene and Plio-Pleistocene may also have facilitated haplotype diversification through changes in forest habitat. In Africa, changes in temperature and precipitation resulted in increased aridity and expansion of the savanna during the glacial maxima (DeMenocal, 1995; 2004; Jacobs *et al*., 2004). Such changes in paleoclimatic conditions and the resultant changes in vegetation have been linked to allopatric speciation within the African fruit bat tribe Myonycterini (Nesi *et al*., 2013) and Scotonycterini (Hassanin *et al*., 2014). According to Baldwin (2015), the major lineages of *H*. aff. *ruber* complex (A, B, C, D) in Ghana diverged during late Miocene and early Pliocene around 5.5 and 4.4 million years ago (Ma) with divergent clades within each lineage branching off around 2.5–1.5 Ma. Further, common ancestors within the extant members of the complex diverged around 3.6 – 1 Ma of the late Pliocene and the Pleistocene.

Morphologically, members of the *H*. aff. *ruber* complex have rounded wing tips with low wing loading and aspect ratio and are predicted to have limited dispersal (Norberg & Rayner, 1987). They roost mostly in caves but also in trees and buildings during the day (Happold & Happold, 2013). In view of the natural history of the species, conditions of Pleistocene and possibly limited tree roosts, and uneven natural distribution of caves, it is reasonable to infer that historically, ancestors of D sub-lineages were widely-distributed over much of its range and were isolated in forest refugia during the past ice-ages.

The Pleistocene forest refuge hypothesis (Haffer, 1982) postulates that forest fragmentation during climatic oscillations of the ice-ages led to the isolation and successive diversification of forest-associated taxa. The Togo highlands, a moist semi-deciduous and montane ravine forest found within a predominately savanna belt is hypothesized to have served as a forest refugium during the climate oscillations of the Holocene (Haffer, 1982). Given the geographical distance between the sampled sites from northern Ghana and Benin (ca. 400 km) as well as the similar distances from these two sites to the Togo hills, similar surrounding vegetation matrix and the general flight capabilities of bats to disperse over long distances; populations from Ghana and Benin clustering in D2 may have diverged from a common ancestor isolated in the Togo highlands. Further, it has been suggested that bats of low wing loading including *H*. aff. *ruber* may be capable of commuting over longer distances than previously assumed (Monadjem *et al*., 2009; Nkrumah *et al*., 2016).

The role of climatic changes during the Pleistocene period in structuring genetic and subsequent speciation events is well studied in rodents (Brouat *et al*., 2009; Nicolas *et al*., 2008, 2011), African herbivores (Flagstad *et al*., 2001; Lorenzen *et al*., 2010) and bats (Flanders *et al*., 2011; Hassanin *et al*., 2014; Lin *et al*., 2014). Further sampling and the use of multiple nuclear and mitochondrial markers may provide insights into nuclear gene flow and genetic structure which may further help resolve the phylogeography of the complex.

5.2 Morphological Variation among *Hipposideros* aff. *ruber*

Morphological variation among sub-lineages of *Hipposideros* aff. *ruber* was studied using geometric morphometric methods (GMM), which are relatively new methods and this study represents the first data for this taxon. Therefore, the discussion is in relation to traditional methods and other bat studies using GMM. Mean centroid size of males was statistically different from female specimens for the D sub-lineages of *Hipposideros* aff. *ruber* complex. Nonetheless, the biological meaning of differences in centroid size among sexes must be interpreted with caution given that these slight differences in skulls do not necessarily represent overall sexual dimorphism for any of the sub-lineages. Typically, members of the traditionally recognized group of *H. caffer* and *H. ruber* are not considered sexually dimorphic (Wright, 2009; Happold & Happold, 2013). Yet, sexual differences associated with echolocation call frequency and morphology (forearm length) have been documented for A, B, C and D lineages of the complex in Ghana (Baldwin, 2015). According to Vallo *et al*. (2011) males of Senegal sub-lineage D1 call at significantly higher frequencies (134–139 kHz) than females (130–135 kHz).

Typical and reverse sexual dimorphism where females are larger than males, have been documented for several families of bats. Among the family Hipposideridae, *H. commersoni* (Cotterill & Fergusson, 1999), *H. diadema* (Kitchener *et al*., 1992), *T. menamena* show patterns of typical sexual dimorphism whereas *T. auritus* and *T. furculus* show reverse sexual dimorphism (Ramasindrazana *et al*., 2013). Further, sexual dimorphism has been documented for other high-duty cycle bats (Siemers *et al*., 2005; Monadjem *et al*., 2010). Generally, sexual selection (Trivers, 1972; Storz *et al*., 2001; Grilliot *et al*., 2009) and male-female competition for food resources (Earhart & Johnson, 1970) have been invoked to explain sexual dimorphism among mammals.

On the contrary, Vallo *et al*. (2011) did not find sexual size differences among the Senegalese specimens of the D-lineages. Such difference in results may be due to differences in methods of measurement used. Traditional methods have consistently showed overlaps in commonly used measurements such as forearm length or condylocanine length for separating members of the Hipposideridae (Hayman & Hill, 1971; Koopman, 1975). Recent attempts at separating the *Hipposideros* aff. *ruber* complex also show subtle morphological differences with considerable overlaps in skull measurement (Vallo *et al*., 2011; Monadjem *et al*., 2013). GMM provides superior resolution with the rigorous statistical support needed for identifying small localized changes useful for discriminating cryptic species (Schmieder *et al*., 2015). Centroid size is also not correlated with shape characteristics and thus provides a confident estimate (Bookstein, 1991). Such improved morphological discrimination using GMM has been documented among other cryptic bat species (Sztencel-Jabłonka *et al*., 2009; Taylor *et al*., 2012) and other traditionally recognized groups (Bornholdt *et al*., 2008; Šrámek & Benda, 2014).

Size and shape separation of the northern and southern population of Ghana and lineages can be interpreted as geographical variations. According to Thorpe (1987), historical processes and adaptations to current ecological conditions can be invoked to explain geographical variation among organisms. Current ecology as predicted by differences in vegetation and temperatures between northern and southern populations of the complex which with other factors determine the abundance and distribution of prey within these habitats, may be responsible for the observed morphological changes to a large extent. Population clustering in sub-lineages D2 and D1 occurs predominantly in woodland savanna habitats. Therefore, overlaps in shape of these lineages may be largely due to similarity in habitat preferences and prey choices. The differences in shape of the zygomatic arch between lineages D3 and D1, D2 presumably reflect slight differences in the diet of sub-lineages of the complex.

The effect of diet preference on the evolution of bat skulls is well documented (Freeman, 1979; 1981, 1995; Nogueira *et al*., 2009). In Ghana, studies on diet of *H.* aff. *ruber* from the southern zone (Buoyem, Forikrom and Kwamang) show seasonal and geographical variation in prey abundance and intake of the major prey items of lepidopterans and coleopterans (Badu, 2013). Such temporal and spatial variation in prey abundance directly influences the dietary habits of bats *(*Zhang *et al*., 2005; Rolfe & Kurta, 2012) which in turn affect the morphology of the skull. Given the prolonged dry season and a unimodal rainfall pattern in northern Ghana compared to the bimodal rainfall pattern in the south, it is speculated that variation in vegetation and its resultant prey abundance and/or choice of prey may influence the morphology of the bats.

Phylogenetic relationships based on mitochondrial DNA showed that populations from southern Ghana (Buoyem, Forikrom, Kwamang) clustering in sub-lineage D3 formed a well-supported clade distinct from northern populations attributable to previously studied sub-lineages D1 and D2 (Figure 5). Size and shape analysis of the cranium of these populations also provide evidence of morphological separation between northern and southern populations (i.e., between sub-lineages D1 and D2, and D3, respectively). Nuclear gene flow among populations from Buoyem, Forikrom and Kwamang sites assigned to sub-lineage D3 in Ghana has been established (Baldwin, 2015) but it remains largely unknown if there is gene flow between populations in lineages D1 and D2.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Identification of organisms to meaningful taxonomic levels and understanding of phylogenetic relationships among local fauna can enhance knowledge of a species and ultimately support management and conservation approaches where necessary. This study reported on the phylogenetic relationships and cranial morphology of Noack's leaf-nosed bat, *Hipposideros* aff. *ruber*.

The West African distributed mitochondrial D lineage consists of three deeply-diverged sub-lineages. Sequence divergences among Ghanaian populations were high compared to other West African populations. Geographically-distant populations were genetically closely-related as compared to geographically-close populations. Geometric morphometric analyses of skulls revealed shape differences within D sub-lineages, mostly in the area of the zygomatic arch as well as differences in centroid size among lineages, sexes and geographical areas. Classification results suggest that individuals clustered in lineages D3 are reliably separable from D2 and D1 as compared to D1 and D2.

6.2 Recommendations

The study highlighted phylogenetic relationships and morphological variation within the mitochondrial D lineage of Noack's leaf-nosed bat, *Hipposideros* aff. *ruber*. However, the phylogeography of the complex is not fully resolved. Use of bi-parentally inherited nuclear and mitochondrial markers may provide additional insight useful for elucidating the phylogeography of the complex. Further, the contribution of additional evidence such as echolocation call frequency to these patterns remains unclear. Thus, there is the need for broader sampling throughout West Africa and the use of multiple data sources including acoustic and other morphological data.

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APPENDICES

Appendix 1: Chemicals, Reagents and Preparation of Standard Solution

Primers for PCR were reconstituted and diluted according to the manufacturer’s protocol. A volume of 90 µl double distilled water (ddH$_2$O) to 10 µl of each of both forward and reverse primers.

Components of PPP Master Mix

PPP Master Mix supplied 2x conc: 150 mM Tris-HCl, pH 8.8 (at 25 ° C), 40 mM $(NH_4)_2SO_4$, 0.02% Tween 20, 5 mM $MgCl_2$, 400 microM dATP 400 microM dCTP, dGTP, 400 microM, 400 microM dTTP, 100 U / ml Taq DNA polymerase, pigments, stabilizers and additives.

Components of a 22.5 µl of PCR reaction mix used in amplification of cytochrome-b mitochondrial gene of *H. aff. ruber*

Reagent	Volume (µl)
PPP Master Mix	12.5
Forward primers	2.0
Reverse Primers	2.0
Double distilled water (ddH$_2$O)	6.0
DNA template	2.5

Sequence of synthetic oligonucleotide sequence primers used for molecular studies

Primers	Sequence (5'-3')
L14724	CGAAGCTTGATATGAAAAACCATCGTTG
H15915	AACTGCAGTCATCTCCGGTTTACAAGAC
F_1	CCACGACCAATGACAYGAAAA
R_1	CCTTTTCTGGTTTACAAGACCAG

Preparation of 1.5% agarose gel

A mass of 1.5g of agarose powder was put into a flask, and 100ml of TAE was added and mixed thoroughly. The mixture was heated in a microwave oven for one to two minutes to dissolve the solute and was allowed to cool down. The gel was cast on a casting tray with a set combs to make wells. Once the gel was ready, a volume of 0.3µl of amplified PCR products was mixed thoroughly with loading buffer with red dye and loaded into the wells for electrophoresis. The gel was electrophoresed in TAE buffer using a mini gel system (BIORAD USA) at 100 volts for 30 minutes and gel photographed under ultraviolet (UV) trans-illuminator (UPC, USA) using a polaroid camera and film type 667 (Polaroid USA).

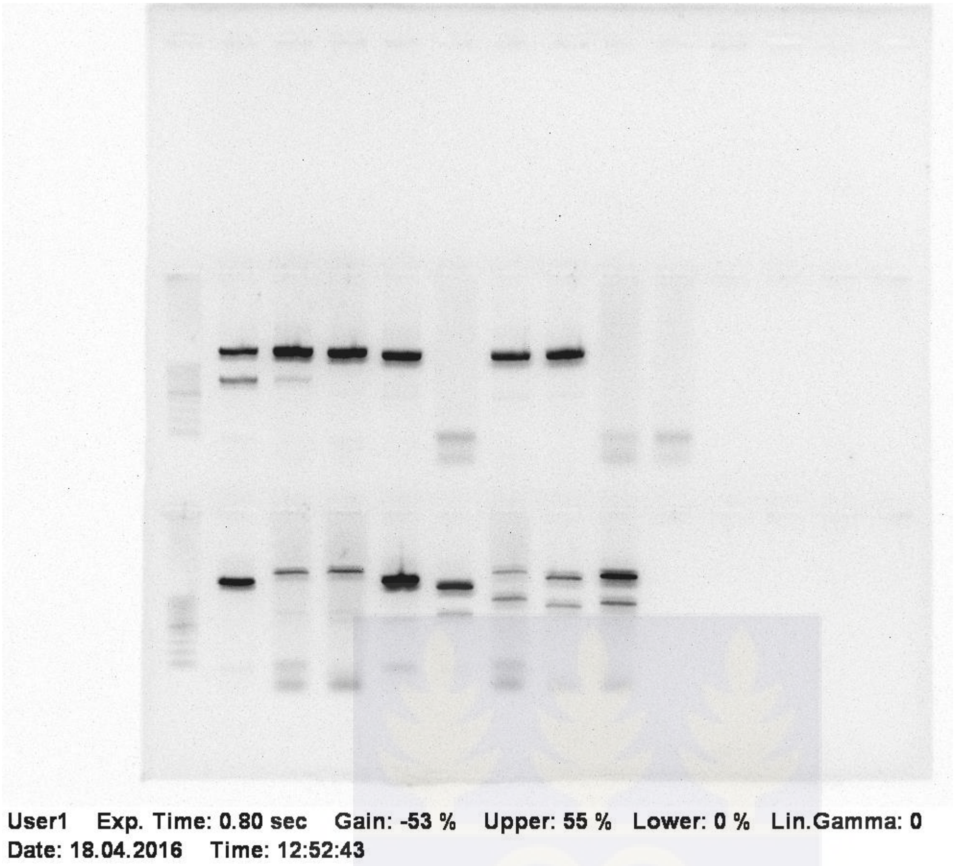


Plate 3: Photograph of red-dye stained agarose gel (1.5%) electrophoretogram of PCR amplified products of *cytochrome b* of *H.* aff. *ruber*. Lane one is a molecular base pair (100 bp) ladder and subsequent bands represent the *cytochrome b* of *H.* aff. *ruber*.

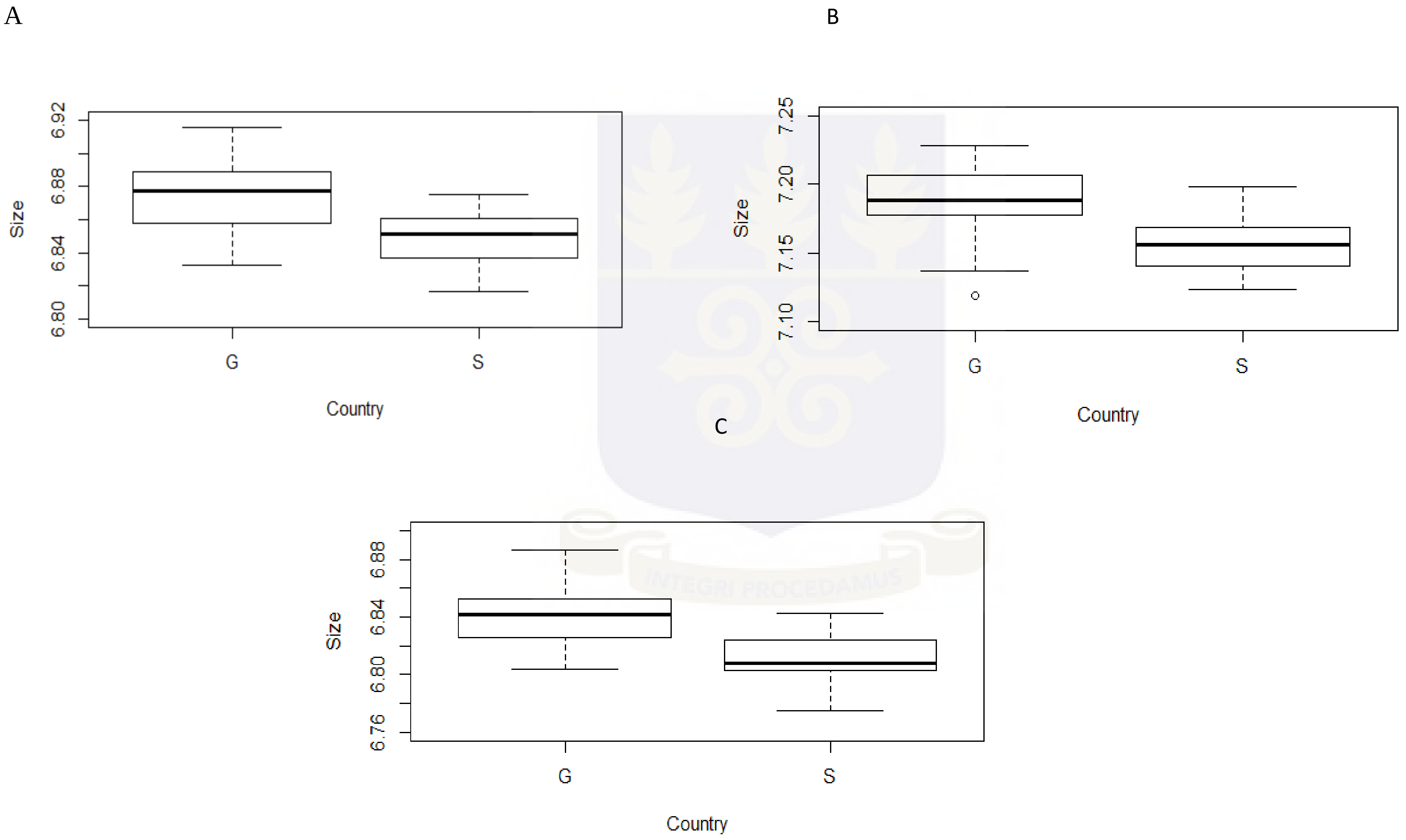
Appendix 2: List of samples, collection numbers, haplotypes, location and Genbank accession numbers for haplotypes included in the study.

Sample	Haplotype	Lineages	Country	location	Accession numbers	Source of sequence
		H. abae	Ghana			This study
IVB S1032		*H. gigas*	Senegal	Dar salam	EU934469	Vallo *et al.* (2008)
IVB S132	Hap_S1	D2	Senegal	Lingué Kountou	HQ343242	Vallo *et al.* (2011)
IVB S281	Hap_S2	D2	Senegal	Simenti	HQ343248	Vallo *et al.* (2011)
IVB S119	Hap_S3	D1	Senegal	Lengue	EU934478	Vallo *et al.* (2008)
IVB S1374	Hap_S4	D1	Senegal	Dar Salam	EU934479	Vallo *et al.* (2008)
IVB S803	Hap_S4	D1	Senegal	Lingué Kountou	HQ343246	Vallo *et al.* (2011)
IVB S899	Hap_S4	D1	Senegal	Dar Salam	HQ343246	Vallo *et al.* (2011)
IVB S16662	Hap_S4	D1	Senegal	Dar Salam	HQ343246	Vallo *et al.* (2011)
IVB S272	Hap_S5	D1	Senegal	Simemeti	EU934481	Vallo *et al.* (2008)
IVB S273	Hap_S6	D1	Senegal	Simemeti	EU934482	Vallo *et al.* (2008)
IVB S275	Hap_S7	D1	Senegal	Simemeti	EU934483	Vallo *et al.* (2008)
IVB S285	Hap_S8	D1	Senegal	Simemeti	EU934484	Vallo *et al.* (2008)
IVB S1561	Hap_S8	D1	Senegal	Dar Salam	HQ343263	Vallo *et al.* (2011)
IVB S820	Hap_S9	D1	Senegal	Dindéfélou	EU934485	Vallo *et al.* (2008)
IVB S825	Hap_S9	D1	Senegal	Dindéfélou	EU934485	Vallo *et al.* (2011)
IVB S1400	HAP_S2	D2	senegal	Dar salam	EU934480	Vallo *et al.* (2008)
NMP 91879	Hap_B1	D2	Benin	Tagayé	EU934476	Vallo *et al.* (2008)
ROM 100516	Hap_IC1	D1	Cote d'Ivoire	Sibabli	EF584226	Lim *et al.* (2008)
PV18	Hap_G1	D3	Ghana	Kwamang		This study
G15_25	Hap_G1	D3	Ghana	kwamang		This study
PV21	Hap_G2	D3	Ghana	kwamang		This study

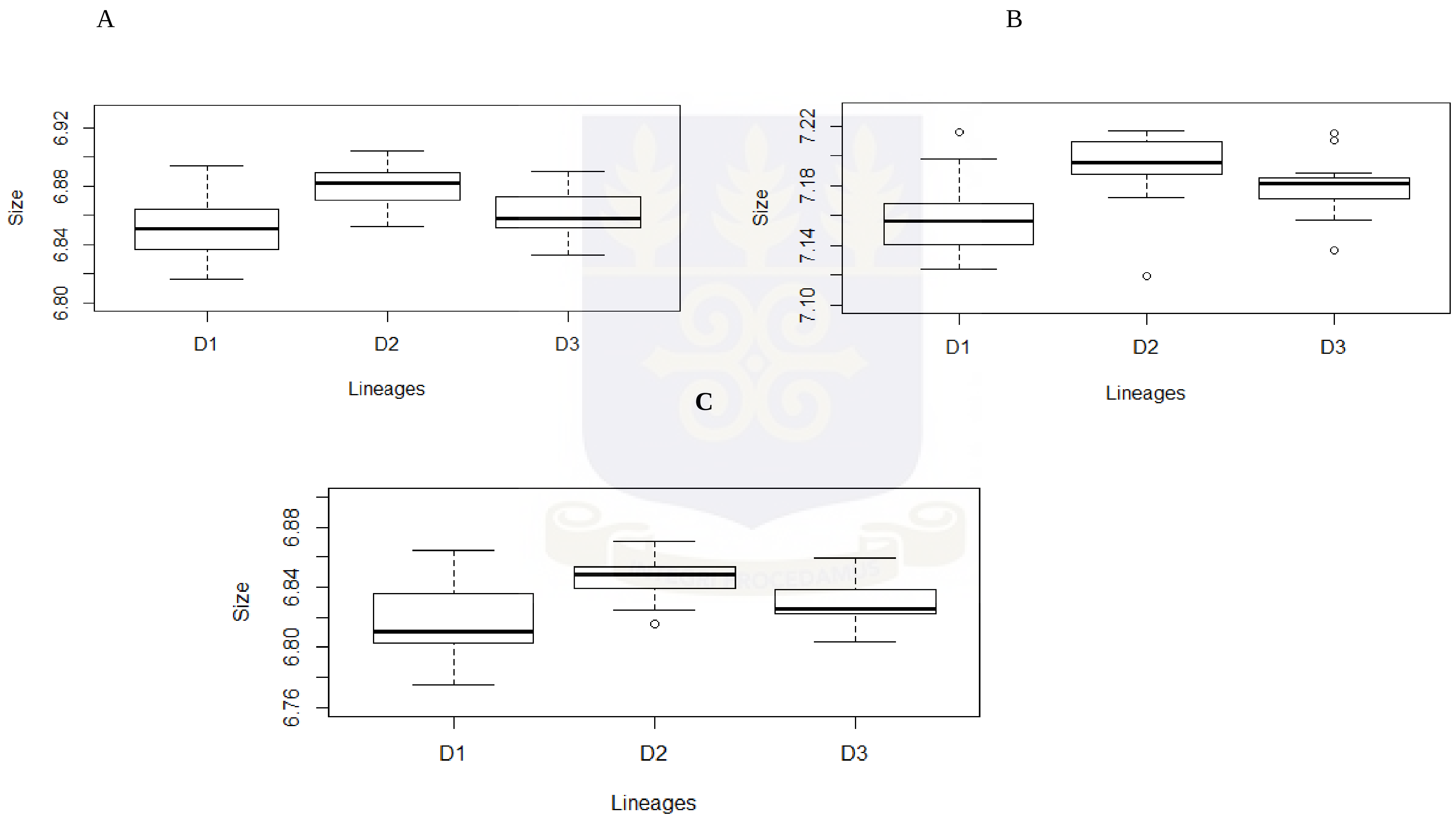
PV24	Hap_G2	D3	Ghana	kwamang		This study
PV29	Hap_G2	D3	Ghana	kwamang		This study
PV31	Hap_G2	D3	Ghana	kwamang		This study
PV32	Hap_G2	D3	Ghana	kwamang		This study
PV55	Hap_G2	D3	Ghana	kwamang		This study
PV26	Hap_G2	D3	Ghana	kwamang		This study
PV56	Hap_G2	D3	Ghana	kwamang		This study
G15_24	Hap_G2	D3	Ghana	Kwamang		This study
G15_124	Hap_G2	D3	Ghana	kwamang		This study
G15_148	Hap_G2	D3	Ghana	Forikrom		This study
G15_149	Hap_G2	D3	Ghana	Forikrom		This study
G15_150	Hap_G2	D3	Ghana	Forikrom		This study
G15_152	Hap_G2	D3	Ghana	Forikrom		This study
IVB PV56	Hap_G2	D3	Ghana	Buoyem	HQ343266	Vallo *et al*. (2011)
PV22	Hap_G3	D3	Ghana	Kwamang		This study
PV25	Hap_G3	D3	Ghana	Kwamang		This study
PV57	Hap_G3	D3	Ghana	Buoyem		This study
PV30	Hap_G3	D3	Ghana	Kwamang		This study
						Vallo *et al*. (2011)
G12_122	Hap_G4	D1	Ghana	Lilikse		This study
G12_124	Hap_G5	D2	Ghana	Lilikse		This study
G12_128	Hap_G6	D2	Ghana	Sankana		This study
G12_336	Hap_G7	D2	Ghana	Sakogu		This study
G12_125	Hap_G7	D2	Ghana	Lilikse		This study
G12_121	Hap_G8	D2	Ghana	Lilikse		This study
G12_330	Hap_G8	D2	Ghana	Sakogu		This study
G12_339	Hap_G8	D2	Ghana	Sakogu		This study
G12_340	Hap_G8	D2	Ghana	Sakogu		This study
G13_111	Hap_G9	D2	Ghana	Gbanwele camp/MNP		This study
G15_41	Hap_G10	D2	Ghana	Brugbani camp/MNP		This study
G15_40	Hap_G11	D2	Ghana	Brugbani camp/MNP		This study
G15_42	Hap_G12	D2	Ghana	Brugbani camp/MNP		This study
G12_332	Hap_G13	D1	Ghana	Sakogu		This study
G12_331	Hap_G13	D1	Ghana	Sakogu		This study
G12_129	Hap_G14	D2	Ghana	Sankana cave		This study
G12_130	Hap_G15	D2	Ghana	Sankana cave		This study
G12_333	Hap_G16	D2	Ghana	Sakogu		This study
G12_345	Hap_G17	D2	Ghana	Sakogu		This study
G12_123	Hap_G18	D2	Ghana	Lilikse cave		This study
IVB	Hap_S10	D1	Senegal	Dar Salam	HQ343253	Vallo *et al*.

S695						(2011)
IVB S702	Hap_S11	D1	Senegal	Dar Salam	HQ343254	Vallo *et al.* (2011)
IVB S900	Hap_S12	D1	Senegal	Dar Salam	HQ343256	Vallo *et al.* (2011)
IVB S1377	Hap_S13	D1	Senegal	Dar Salam	HQ343260	Vallo *et al.* (2011)
ROM 100518	Hap_IC2	D1	Cote d’Ivoire	Sibabli	HQ343264	Vallo *et al.* (2011)
IVB PV59	Hap_G19	D3	Ghana	Buoyem	HQ343265	Vallo *et al.* (2011)

Appendix 3a: Boxplot of the skull size variability among *Hipposideros* aff. *ruber* from Ghana (G) and Senegal (S) based on dorsal (A), lateral (B) and ventral (C).



Appendix 3b: Boxplot of the skull size variability among the D sub-lineages of *Hipposideros* aff. *ruber* based on dorsal (A), lateral (B) and ventral (C)views

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