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Contribution towards the development of a DNA barcode reference library for West African mammals

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DNA barcoding is a widely used molecular approach for species cataloging for unambiguous identification and conservation. In the present study, DNA barcoding of some West African mammals were performed with six new mitochondrial CO1 sequences for *Civettictis civetta, Tadarida nigeriae, Orycteropus afer, Heliosciurus gambianus, Equus africanus asinus* and *Funisciurus anerythrus* which are absent in public databases such as BLAST/NCBI and BOLD. Sequence identifications were made by comparing unknown sequences against the DNA barcodes of known species through distance-based tree construction and alignment probing. The sequences have been deposited to GenBank/NCBI.

Key words: mtDNA, West African mammals, conservation, biodiversity.

INTRODUCTION

West Africa is endowed with rich flora, fauna and variety of ecosystems. These ecosystems contain wide variety of endemic species with highly restricted ranges within the region. In Nigeria, for instance, there are 290 mammals of which one species is critically endangered, 13 are endangered, 16 are vulnerable, and 10 are near-threatened (IUCN, 2012). However, many species present in this diverse geographic area have not been classified and this has hampered progress in understanding species richness and for developing strategies for conservation efforts (Burgess-Herbert et al., 2010). The nomenclature of organisms is essential for bridging taxonomical research between other disciplines of science catering for human welfare. However, this process to categorize biological diversity is greatly affected by the lack of taxonomic expertise (Ke and Loren, 2006).

Since its initial proposal as a tool for rapid identification of species, a technique using a primer set to amplify a 648-base pair (bp) region of the mitochondrial cyto-chrome-*c* oxidase subunit 1 (*COI*) gene to ensure rapid and accurate identification of a broad range of biological specimens (Hebert et al., 2003), DNA barcoding has gained considerable validation. Among terrestrial verte-brates, this approach has been shown to be effective in the identification of amphibians (Smith et al., 2008), North American birds (Hebert et al., 2004; Kerr et al., 2007) Neotropical birds (Kerr et al., 2009) and Neotropical small mammals (Clare et al., 2007). The basic idea behind DNA barcoding is the comparison of nucleotide sequence of a standard gene region of an unknown species with a reference library of known DNA barcode to establish a species identity for the query. Ergo, DNA barcoding cannot be used for species identification when there are no barcode records for the query sequence in the reference library (Wong et al., 2011). Development of a reference DNA barcode library of all the known species identified by taxonomists are the first and most important step to establish DNA barcoding as a tool for accurate species identification. Due to the lack of a DNA

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Table 1. List of mammal species used in the present study.

West African mammals species studied	Collection locality	GenBank accession number for CO1
Carnivora: Viverrinae		
Civettictis civetta	Otuocha	JX426124
Felidae		
Felis catus	Orba	JX426133
Chiroptera: Molossidae		
Tadarida nigeriae	Obimo	JX426125
Artiodactyla: Bovidae		
Tragelaphus scriptus	Uvuru	JX426130
Bos taurus	Nru	JX426135
Rodentia: Sciuridae		
Heliosciurus gambianus	Uvuru	JX426127, JX426128
Funisciurus anerythrus	Uvuru	JX426129
Muridae		
Rattus rattus	Nsukka	JX426131
Tubulidentata: Orycteropodidae		
Orycteropus afer	Uvuru	JX426126
Perissodactyla: Equidae		
Equus ferus caballus	Obollo afor	JX426134
Equus africanus asinus	Obollo afor	JX426132

reference library for the West African mammals, the latter have scarcely been identifiable (Burgess-Herbert et al., 2010).

Biodiversity conservation in the fields of ecology, evolutionary biology, agriculture, economics among others relies on accurate species identification, which is pivotal to the basic aspect of recognizing and describing biodiversity. For instance, unlike the usual classification based on morphological aspects according to taxonomic studies involving ingenuity of taxonomists and trained technicians to identify taxa accurately with accumulation of special skills over the years through experience is the use of molecular instead of morphological data for identifying taxa, which has long been a fundamental idea of many biologists (Busse et al., 1996; Blaxter 2003). Progressive achievements in DNA-sequencing technologies have enabled researchers studying biodiversity to conduct simple, cost-effective and rapid DNA analyses. This progress in biotechnology, and the taxonomy crisis itself, played a large role in the creation of DNA barcoding (Jinbo et. al., 2011).

A good number of taxonomists are alarmed that DNA barcoding will compete with the age long traditional taxonomic studies for example Ebach and Holdrege (2005a, b). However, DNA barcoding is inseparably linked to taxonomy, a potent tool that compliments taxonomic studies (Schindel and Miller 2005; Hajibabaei et al., 2007). The integration of various types of data, such as morphological, ecological, physiological and molecular data, including DNA barcodes, will improve species finding and description practices (Waugh, 2007; Padial et al., 2010). This synergic approach will be supported by various biodiversity databases (Jinbo et. al., 2011). Nevertheless, Judging from its wider recognition now as a veritable identification tool, DNA barcoding has become a very important type specimens description in the developed parts of the world unlike parts of the world like Africa. The present work was designed in such a way to generate DNA barcode sequences of some of the West African mammals, which may enhance building a reference DNA barcode library of Western Africa.

MATERIALS AND METHODS

Eleven (11) mammalian species (Table 1) collected from various parts of West Africa were selected for the study after confirming their taxonomy. DNA was extracted from alcohol preserved muscle tissue (~25 mg) by using Qiagen DNeasy Blood and Tissue kit. Universal primers were used in the present study for amplifying CO1 gene (Ivanova et al., 2007). Polymerase chain reactions (PCRs) were performed in 25 µl reactions consisting of 2.5 µl each of 10x PCR buffer, $MgCl_2$ (25 mM) and 0.5 µl dNTPs (2 mM), 0.25 µl of each primer (10 µM), 1 µl of Taq DNA polymerase, 14 µl of dH_2O and 4 µl of template DNA (10-20 ng) in a Thermocycler (ABI 9700). The following thermo cycling conditions were used for amplifications: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 52°C for 40 s, 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were visualized on 1% agarose gels and the most intense products were purified using Exo Sap IT (USB). Bidirectional sequencing was performed using the PCR primers and products were labeled with BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc.) and sequenced in an ABI 3730 capillary sequencer following manufacturer's instructions. The sequences were aligned using ClustalW and potentially misaligned sequences were excluded. The

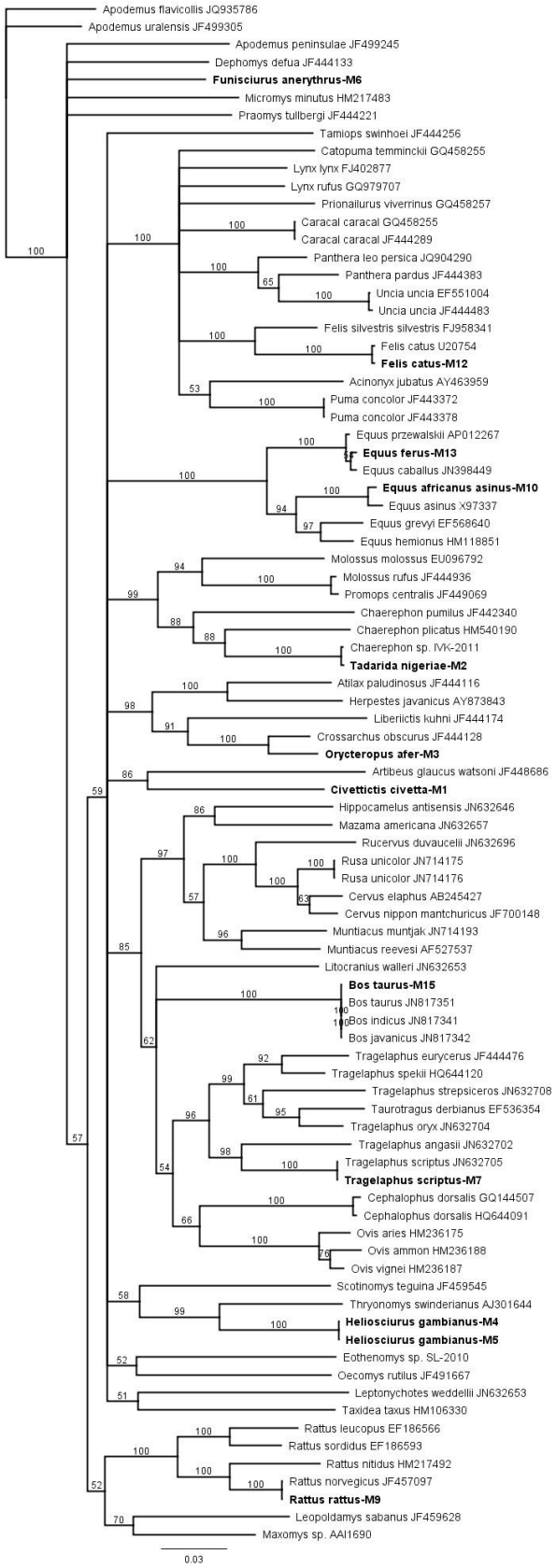

Figure 1. Neighbour joining tree based on *CO1* gene (500 bootstrap replicates).

extent of sequence differences between species was calculated averaging pair-wise comparisons of sequence differences across all individuals. Pairwise evolutionary distance was determined by the Kimura-2-parameter method using the software programme Mega 5 (Tamura et al*.,* 2011). The number of polymorphic sites and nucleotide diversity (pi), nucleotide composition and number of transition and transversion between species were determined. Gaps were considered as missing data on the phylogenetic reconstructions. Neighbour Joining (NJ) tree was constructed to show intraspecific and interspecific relationships among the new sequences and related sequences in GenBank. The new sequences are deposited in GenBank. (Accession numbers are given in Table 1).

RESULTS AND DISCUSSION

All samples were successfully sequenced for CO1 using the forward and reverse primers to obtain robust forward and reverse sequences of approximately 582 bp. No insertions, deletions or stop codons were observed in any sequence of CO1. After alignment, there were 336 common sites in the partial sequence of CO1 genes including inserted gaps used for this analysis. No areas of uncertain alignment were identified. After filtering, there were 246 (42.2 %) variable sites and all of them were phylogenetically distinct. Based on analyses of the estimates of evolutionary divergence between sequences, the nucleotide composition was extremely guanine poor T = 30.1%, A= 27.3%, C = 26.5% and G = 16.2% with 42.7% GC content. The overall mean divergence (d) of the studied samples was 0.227 (Table 2), conserved sites 356, variable sites 226, parsimony informative sites 192 and singleton sites 34. The sequence comparison of CO1 sequence data of West African mammals revealed that six among the 11 species included in the present study were not represented earlier in public data bases (BOLD and GenBank). They are *Civettictis civetta, Tadarida nigeriae, Orycteropus afer, Heliosciurus gambianus, Equus africanus asinus* and *Funisciurus anerythrus.* Neighbour joining (NJ) tree based on CO1 sequence generated in the present study as well as the sequences of all the West African mammals available with the GenBank are represented in Figure 1.

C. civetta (Schreber, 1776) is commonly known as African civet and is listed as least concern as the species has a wide distribution range with a variety of habitats and present in many protected areas. It may be undergoing some localized declines due to hunting and might be rendered more vulnerable in areas where preferred bush meat becomes scarce (IUCN, 2012). *T. nigeriae* (Thomas, 1913) is commonly known as Nigerian Free-tailed Bat feeding primarily on arthropod species. There are 106 species of bat reported from West Africa including fruit bats and insectivorous bats (Okafor et al*.,* 2004). Many of the latter are house dwelling while all of the former are predominantly found in the wild. The aardvark *O. afer* (Pallas, 1766) is a medium-sized,

Table 2. Estimates of evolutionary divergence between sequences.

Species	1	2	3	4	5	6	7	8	9	10	11	12
Civettictis civetta-M1	0.000											
Equus africanus asinus-M10	0.249	0.000										
Felis catus-M12	0.226	0.287	0.000									
Equus ferus-M13	0.234	0.100	0.266	0.000								
Bos taurus-M15	0.253	0.214	0.302	0.229	0.000							
Tadarida nigeriae-M2	0.221	0.223	0.246	0.199	0.235	0.000						
Orycteropus afer-M3	0.194	0.231	0.241	0.256	0.220	0.215	0.000					
Heliosciurus gambianus-M4	0.239	0.229	0.278	0.232	0.227	0.245	0.238	0.000				
Heliosciurus gambianus-M5	0.239	0.229	0.278	0.232	0.227	0.245	0.238	0.000	0.000			
Funisciurus anerythrus-M6	0.240	0.260	0.285	0.240	0.209	0.236	0.221	0.205	0.205	0.000		
Tragelaphus scriptus-M7	0.258	0.248	0.281	0.240	0.163	0.229	0.185	0.204	0.204	0.216	0.000	
Rattus rattus-M9	0.228	0.262	0.250	0.239	0.221	0.227	0.206	0.214	0.214	0.163	0.241	0.000

burrowing, nocturnal mammal native to Africa. It is the only living species of the order Tubulidentata although other prehistoric species and genera of Tubulidentata are known (IUCN, 2012). *E. africanus asinus* (Linnaeus, 1758) commonly known as African Ass is a domesticated member of the Equidae or horse family. Small numbers of donkeys are kept for breeding or as pets in developed countries (IUCN, 2012). *H. gambianus* (Ogilby, 1835) is commonly known as Gambian sun squirrel and believed to be a complex of several similar species (IUCN, 2012). This species is typically associated with savanna woodlands. Populations have also been observed within riparian forest and in savanna areas. It is generally absent from closed forest habitats. This species is commonly found in agricultural areas, especially oil palm plantations. Animals are diurnal, solitary and predominantly arboreal. The sequence obtained in the present study may serve as a reference material for further research to address the question of complex species status of this group. *F. anerythrus (*Thomas, 1890) is commonly known as Redness Tree Squirrel or Thomas's Rope Squirrel. This species is generally found in lowland tropical moist forest throughout much of the range, but has also been reported from gallery forest. It can be found in secondary habitats. Animals are usually found singly or in pairs (IUCN, 2012).

The West African population present in Benin and Nigeria might represent a distinct species for which the sequence obtained in the present study may help further taxonomic studies needed to resolve this question. Limitations in the available specimens and molecular data have prevented us from covering major portion of the West African mammal species in this study. It is also necessary to continue with studies focused on mammals throughout their distribution with the additional aim of investigating the phylogenetic and taxonomic status in various habitats. This will help in proposing conservation policies for species associated with these habitats in West Africa. Further molecular works and their cross references with morphological and ecological studies will provide new insights into the phylogeny and taxonomy of West African mammals.

Ethical considerations

All the ethical issues (including prevailing laws on the collection of samples from the wild in Nigeria) have been completely observed by the authors.

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