SYSTEMATICS, POPULATION STRUCTURE AND DEMOGRAPHY OF THE AFRICAN DWARF CROCODILE (*OSTEOLAEMUS* SPP.): A PERSPECTIVE FROM MULTIPLE SCALES

by

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A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy
Department of Ecology and Evolutionary Biology
2009

This thesis entitled:
Systematics, Population Structure And Demography Of The African Dwarf Crocodile (*Osteolaemus* Spp.): A Perspective From Multiple Scales

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Eaton, Mitchell Joseph (Ph.D., Ecology and Evolutionary Biology)

Systematics, Population Structure And Demography Of The African Dwarf Crocodile (*Osteolaemus* Spp.): A Perspective From Multiple Scales

Thesis directed by Professor Andrew P. Martin

Wildlife harvest is the most geographically widespread form of resource extraction in equatorial forests. The illegal trade in wildlife is worth billions of dollars annually, with some estimates placing it second only to the global trafficking in illicit drugs. In competition with the commercial harvest, many rural inhabitants of tropical forests subsist on wildlife as a protein and economic resource. Lack of understanding of the biology of the system and the inability to implement management controls are cited as the two main impediments to sustainable-use practices in the tropics. To achieve long-term sustainability of wildlife resources, we seek an integrated understanding of the dynamics regulating resource populations and the dynamics of extractive activities influencing the fate of population trajectories. To pursue these goals, my research focuses on a poorly known reptile in tropical Africa that is widely consumed in rural diets but threatened by commercial hunting.

African dwarf crocodiles (*Osteolaemus* spp.) remain an important subsistence food resource throughout Central and West Africa, but are now threatened by over-harvesting. The ability to address harvest issues and conservation priorities for crocodile populations is limited by a lack of knowledge of their biology, ecology and evolutionary history. My research approaches this problem from multiple perspectives. I first seek to resolve an 80-year-old debate on the taxonomy of the dwarf crocodile across its range, which has impeded our understanding of evolutionary significant units (ESUs) and the magnitude of the threats facing regional populations. Novel methods for monitoring the trade in crocodiles and other bushmeat species are needed to understand the magnitude of wildlife use in the tropics. I employed a molecular method to identify commonly hunted African wildlife species. I then attempt to gain inference on population genetic structure, connectivity and landscape factors influencing individual movements at the local and regional scales. At the population level, I used field surveys and capture-recapture methods to estimate demographic parameters and model population growth, incorporating empirical estimates of harvest rates and size-selection bias to evaluate their impact on long-term viability. I offer recommendations for new conservation management units, new techniques for monitoring the bushmeat trade and suggestions for spatial harvest management.

Acknowledgements

This research could not have been completed without the assistance of many institutions and individuals contributing financial backing, intellectual ideas, moral support and help in the field.

Andrew Martin has been my champion from the time he accepted me into his lab through the completion of this dissertation. Andy allowed me to conduct this research largely on my own, trusting that I would eventually find the right path while offering a great deal of support and feedback anytime I asked for it. John Thorbjarnarson envisioned this project on Central African crocodiles early-on and invited me to join him on an expeditionary pilot study to Congo and Gabon in 2003. Together, we outlined the structure of this research project and wrote grants to fund it; John provided ideas and support throughout the endeavor. I offer my gratitude to my other committee members, Sharon Collinge, Alan Townsend and J. Terrence McCabe, for their thoughtful contributions and discussions.

The Wildlife Conservation Society (WCS) and the governments of the Republics of Congo and Gabon facilitated field research. I especially thank the directors of Loango NP (Tomo Nishihara), Mayumba NP (Richard Parnell), Ivindo NP (Ruth Starkey and Nigel Orbel) and the Lac Télé Community Reserve (Hugo Rainey and Emile Ngouaka) for help with logistics. In Gabon, Edward Truter, Ross Spoor, Philipe DuPlessis, Alan Mougoula, Sophiano Etouck, Pierre Bokosso, Ellen Bean, Guy Serge Mbina, Brice Oguendo, Dimitri Mavoungou, Basil Koumba, Tim Collins and the rest of the Loango staff helped with field work and logistics. In Congo, I thank Faustin Otto, Roger Mobongo, Gerard Bondeko, Emanuel Balipe, Nazaire Massamba, and the rest of the Lac Télé staff for their support, hard work and dedication to conservation and monitoring in the Reserve. WCS country directors Paul & Sarah Elkan, Bryan Curran and Lee White were instrumental in securing permits and ensuring the research happened.

Field work in Central Africa and genetic laboratory research are very expensive and I was offered financial support from many sources. The American Museum of Natural History, under the invitation of Dr. George Amato, supported all of the lab work presented here. It is unlikely I would have been able to generate this level of support on my own, so I am grateful for the opportunity. Field research was funded by the National Geographic Society's Committee for Research and Exploration, Lincoln Park Zoo's Conservation and Science Department, the Rufford Foundation Small Grants Program, the University of Colorado's Natural History Museum and the Rozella Smith Fellowship. Logistic and additional financial support in the field was provided by Rombout Swanborn's Société de Conservation et Développement (SCD) and the WCS Congo and Gabon Programs.

Many others contributed to this project in various ways. Matt Shirley provided tissue samples from West Africa and several constructive discussions; colleagues and friends from AMNH including Sergios Kolokotronis, Susan Perkins, Matt Leslie, Isabela Diaz, Craig Starger, Greta Meyers, Cristina Pomilla, Angelica Cibrian, Martin Mendez and Eugenia Naro-Marciel welcomed me into the lab and were exceedingly generous in sharing their knowledge; Brett Melbourne provided guidance on constructing models in R; Natalie Robinson helped in the early stages of GIS manipulations. Most significantly, Ellen Bean has continually shared her keen judgment and editorial eye. She contributed immensely with everything from fund raising to field work and this research would have amounted to a fraction of what is presented without her input. I cannot thank her enough.

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Chapter 1. Species-level diversification of African dwarf crocodiles (Genus *Osteolaemus*): a geographic and phylogenetic perspective

Introduction

The African dwarf crocodile (Crocodylidae; *Osteolaemus tetraspis* ssp.) is a small, secretive crocodilian restricted to wetlands in closed-canopy tropical forests of Central and West Africa (Fig. 1). The dwarf crocodile is an important food and economic resource for rural inhabitants of Central Africa. In areas of abundant swamp and seasonally-flooded forest, dwarf crocodiles constitute as much as 25% of non-fish biomass in the 'bushmeat' harvest (Auzel and Wilkie 2000, M. Eaton, unpub. data). Human population growth, modern hunting techniques and improved transportation infrastructure in Central Africa have resulted in increased commercial trade of dwarf crocodiles and other bushmeat species within the region and, increasingly, to satisfy expatriate markets outside of Africa (Milius 2005). The dwarf crocodile is listed as an Appendix I species under the Convention on International Trade in Endangered Species (CITES) and vulnerable (VU A2cd) in the IUCN Red Book due to a suspected decline in population size caused by habitat loss and exploitation (Crocodile Specialist Group 1996; Ross 1998). Most ecological studies of the dwarf crocodile have been of limited scope or duration (Kofron and Steiner 1994; Luiselli *et al.* 1999; Riley and Huchzermeyer 1999, 2000; Pauwels *et al.* 2007) and the few genetic studies to date have used small sample sizes from animals of unknown origin (Densmore and White 1991; Brochu and Densmore 2000; Ray *et al.* 2000).

The taxonomy of the dwarf crocodile has been debated since the early 1900's (Schmidt 1919; Chabanaud 1920; Mertens 1943; Inger 1948; Wermuth and Fuchs 1983; Densmore and White 1991; Ray *et al.* 2000; Brochu 2007). The type specimen of *Osteolaemus tetraspis*, described by Cope (Academy of Natural Sciences of Philadelphia 1860), originated from the "Ogobai" (= Ogooué) River basin in Gabon (Fig. 1). Almost 60 years later a new genus and species of dwarf crocodile, *Osteoblepharon osborni*, was described from the Ituri forest of what is now eastern Democratic Republic of Congo (Schmidt 1919; Fig. 1). The genus *Osteoblepharon* was later considered to be unwarranted by several authorities (Werner 1933; Mertens 1943; Inger 1948). These authors were in agreement, however, that the eastern form should be considered a valid and separate species, *Osteolaemus osborni* Schmidt. Subsequently, the genus *Osteolaemus* was reduced to a single species, with differences in morphology rejected as ontogenetic or intraspecific, and two allopatric sub-species designated as *O. t. osborni* (Congo Basin) and *O. t. tetraspis* (West Africa) (Wermuth 1953; summarized in Savage 1956). Some authors have suggested that variations observed in *Osteolaemus* represent a cline and that the recognition of sub-species is not merited (King and Burke 1989; Ross 2006).

In the most comprehensive molecular examination of *Osteolaemus* to date, ten samples (only two of known origin) revealed two clades with relatively high levels of genetic divergence in a 350 bp concatenated fragment of the mitochondrial ND6 and cytochrome-*b* genes (Ray *et al.* 2000). All individuals used in the study were classified morphologically as *O. t. tetraspis*, and the authors predicted that higher levels of divergence between subspecies would eventually be discovered. Recent

treatment of *O. osborni* as a full species is based on a morphological assessment in which differences of four cranial characters are equivalent or greater than in currently recognized crocodilian species (Brochu 2006; McAliley *et al.* 2006; Brochu 2007). McAliley et al. (2006), examining the phylogeny of the genus *Crocodylus*, recognized both species of *Osteolaemus* in a morphological assessment but were only able to sequence samples of *O. tetraspis* for their molecular analysis. Thus, the resurrection of *O. osborni* as a full species has not yet been evaluated by molecular analysis using multiple gene regions and sufficient individuals of known origin.

Confusion surrounding the diagnosibility and significance of morphological characters (e.g. Wermuth 1953) and the lack of specimens of known origin has prevented confirmation of evolutionary distinct lineages of the dwarf crocodile. This is problematic because effective conservation and management of endangered or cryptic species depends on accurate taxonomy (Goldstein *et al.* 2000; Frankham *et al.* 2002, p.16; Coulon *et al.* 2006; Witt *et al.* 2006). Defining species boundaries is also a key to our understanding of broad-scale evolutionary patterns and resolving primary units of study in ecology, biogeography and conservation biology. An inability to differentiate between population boundaries (which delimit demographic processes) and species boundaries (defining the limit of evolutionary processes) will obscure inferences gained from studies at the population or species level by failing to resolve the basic units of evolution (Sites and Marshall 2003).

In this study, I sequenced three mitochondrial (mtDNA) and two nuclear (nuDNA) gene fragments from numerous individuals sampled from the putative range of *O. t. tetraspis* and *O. t. osborni* to test hypotheses about the phylogeny,

phylogeography and taxonomy of African dwarf crocodiles. Using molecular data and a limited morphological analysis I test a hypothesis of independent evolution of geographic lineages against a null hypothesis that the dwarf crocodile represents a single, panmictic species across forested Africa. A priori geographic groups include the Congo Basin (Rep. of Congo and Dem. Rep. of Congo), the greater Ogooué Basin (Gabon, Equatorial Guinea and southern Cameroon) and western Africa (west of the Cameroonian Highlands) (Fig. 1). These results provide convincing evidence for three evolutionary divergent lineages of *Osteolaemus* and I propose a new hypothesis that each is distinguishable as a unique species representing the smallest diagnosable phylogenetic unit (Cracraft 1983; Tattersall and Mowbray 2005).

Materials and methods

Sampling, DNA sequencing and alignment

Samples from wild-caught and locally hunted crocodiles were collected by MJE in Loango N.P. and Mayumba N.P. in the Republic of Gabon and from the Lac Télé Community Reserve in the Republic of Congo (LTCR) (Fig. 1). Wild-caught animals were captured, sexed, measured, marked and released as part of a broader research program on the ecology and population dynamics of Central African crocodiles (Eaton 2006). Caudal scute clippings, used to individually mark animals, were collected for use in genetic analyses. A sub-sample of tissue was stored in either 95% EtOH or a 10% buffered solution of ethylenedianime-tetraacetaic acid (EDTA) and dimethylsulfoxide (DMSO) and maintained at room temperature; excess tissue was air dried and stored individually in desiccant at room temperature. I obtained additional dried skin samples of the holotype and a paratype of *O. t. osborni*

(AMNH 10082 and 10083, respectively. See Schmidt 1919) collected in the Ituri Forest of the Democratic Republic of Congo, and one sample collected in Cameroon (AMNH 75421, locale unknown). A collaborator (M. Shirley, Univ. of FL, USA) provided contemporary samples (n = 12) collected from the wild and from zoos in Ghana and Ivory Coast (see Fig. 1 and Table 1 for samples and localities).

Total genomic DNA was extracted using DNEasy tissue kits (Qiagen, Valencia, CA), following a modified protocol of the manufacturer in which dried tissue and museum samples were digested for 48 h and an additional 20 µl of proteinase-K was added after 24 h. Any undigested tissue was pelleted by centrifuge and the supernatant transferred to a new tube to complete the extraction. Following the addition of ethanol, the extraction was cooled to -4°C for 30min before being passed through a Qiagen spin column. Final elution buffer was heated to 70°C prior to an elution in 70 µl ddH_20.

From genomic DNA template, I amplified and sequenced fragments of mitochondrial cytochrome *b*-tRNA^{Thr,Pro,Phe}-control region (cyt*b*-CR), 12S and cytochrome *c* oxidase I (CO1) genes and nuclear lactate dehydrogenase A (LDH-A) and recombination-activating gene 1 (RAG-1). Internal primers were required for sequencing RAG-1 gene fragments and for COI fragments of degraded museum samples (see Table 2 for genes, primers, and sample sizes). Polymerase chain reactions (PCR) were performed in a volume of 25 µl and contained 2.0 µl genomic DNA (~25 ng/µl), 1X PCR Buffer (Fisher Scientific), 0.24 µM dNPTs, 15 ng BSA, 1 U *Taq* polymerase (Fisher Scientific), and 0.4 µM of each forward and reverse primer (Integrated DNA Technologies, Coralville IA). Amplification was performed in an

Eppendorf Mastercycler gradient thermocycler (Brinkmann Instruments, Westbury, NY). PCR of published primers followed thermocycling conditions provided in the

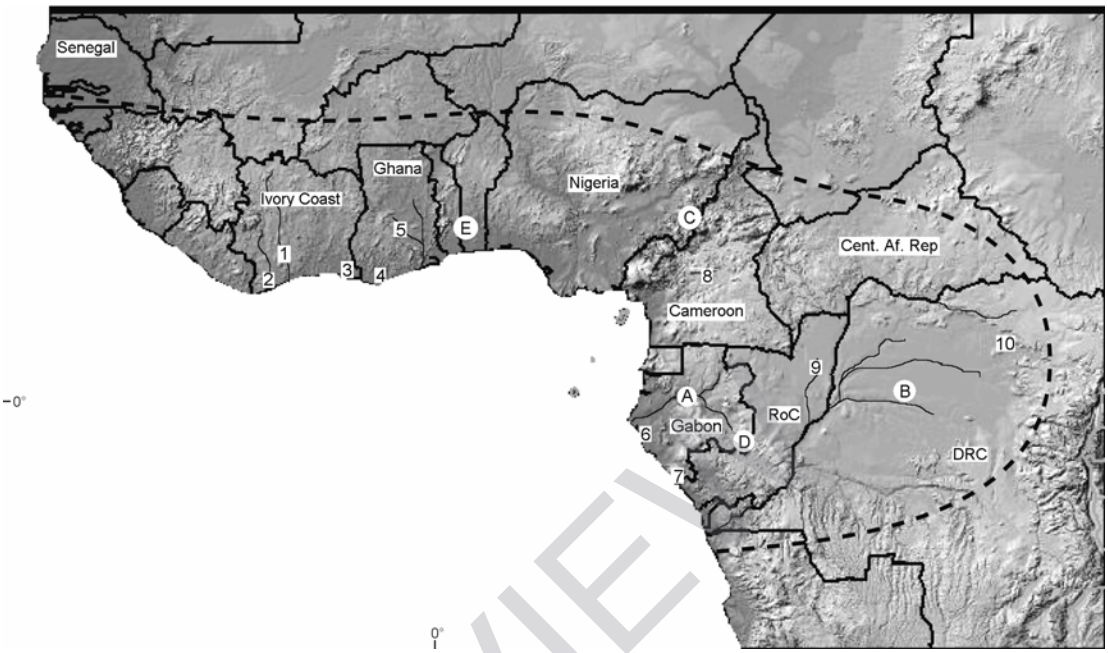

Figure 1. Shaded relief map of Central and West Africa showing geographic features (letters in circles) and sampling localities (numbers in squares) included in this study. The estimated range of *Osteolaemus* is indicated by the dotted line. Major geographic regions considered here include **West Africa :** Sassandra River IC [1], San Pedro River IC [2], Abi Lagoon IC [3], Amansuri Wetlands GH [4], Owam River GH [5]; the **Ogooué Basin (A):** Loango Nat'l Park GA [6], Mayumba Nat'l Park GA [7], unknown locality Cameroon [8]; and the **Congo Basin (B):** Lac Télé Community Reserve RoC [9], Ituri Forest DRC [10];. IC = Ivory Coast, GH = Ghana, GA = Gabon, RoC = Republic of Congo, DRC = Democratic Republic of Congo. Additional geographic features represented are: Nigerian-Cameroon Highlands (C), Batéké Plateau (D), and the Dahomey Gap (E).

original publication with the exception of annealing temperature for 12S A-L/B-H (50.5°) and cycle number (35 cycles) for LA17-F1/R1 (Table 2). Cycling conditions for CoxI(L2/H2) were 95º for 1 min, 50º for 1 min, and 72º for 30 sec (35 cycles) (C. Borgwardt, pers. comm.). Conditions for internal COI primers (CoxIH2/ COIr-ot1; COIf-ot1/ COIr-ot2; COIf-ot2/ CoxIL2) were 92º for 30 sec, touchdown annealing of 50º for 45 sec (8 cycles), 48º for 40 sec (10 cycles) and 46º for 40 sec (10 cycles), followed by an extension at 72º for 40 sec. Conditions for amplifying overlapping

RAG-1 fragments (RAG1L1/R1; RAG1L3/R5) were 95º for 1 min, 61º for 1 min, 72º for 1 min (35 cycles), and for internal primers (RAG1int-F/R) were 92º for 30 sec,

Table 1. Collection localities for samples used in this study

Region	Site	Coordinates	N[a]
Congo Basin	Lac Télé Community Reserve	N1º20' E17º28'	34
	Ituri Forest	N2º21' E26º26'	2
Ogooué Basin	Loango National Park	S2º7' E9º30'	39
	Mayumba National Park	S3º34' E10º53'	7
	Cameroon	Unknown	1
West Africa	Sassandra River IC	N6º 11' W6º 59'	1
	San Pedro River IC	N4º 59' W6º 38'	1
	Abi Lagoon IC	N5º07' W3º01'	1
	Amansuri Wetlands GH	N5º 00' W2º 35'	2
	Owam River GH	N6º 57' W1º 11'	1
	Abidjan Zoo, IC	Unknown	3
	Kumasi Zoo, GH	Unknown	1
	Accra Zoo, GH	Unknown	2

[a] maximum number of individuals sequenced for any given gene fragment

touchdown annealing of 63º for 45 sec (8 cycles), 61º for 40 sec (10 cycles), and 59º for 40 sec (10 cycles), followed by an extension of 40 sec at 72º. Cyt-*b*/CR (14943L/15789H) conditions were 92° for 30 sec, 51° for 45 sec, and 72° for 40 sec (30 cycles). PCR products were purified using AMPure beads and following the manufacturer's protocols (Agencourt, Beverly, MA). All gene regions were sequenced in both directions using BigDye 1.1 chemistry (Perkins-Elmer, Foster City, CA) on an ABI 3730xl (Applied Biosystems, Foster City, CA). Forward and reverse sequences were assembled into contigs, edited with Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI) and verified by eye. Contig sequences were aligned using ClustalW as implemented in MEGA 4.0 (Tamura *et al.* 2007).

Data Analyses

Tree-Based Phylogenetic Analysis

I calculated corrected genetic distances (K2P, Kimura 1980) between and within each aggregated population included in this study. Three methods of phylogenetic inference were then used to evaluate hierarchical relationship among dwarf crocodiles: parsimony (MP), maximum-likelihood (ML) and Bayesian inference (BI).

Table 2. Gene fragments, sequence length, sample sizes and primer information

Gene Region	N[a]	Size (bp)	Primer		Source
12S	80	407			
			12SA-L	AAACTGGGATTAGATACCCCACTAT	(Schmitz *et al.* 2003)
			12SB-H	GAGGGTGACGGGCGGTGTGT	(Schmitz *et al.* 2003)
CO1	82	565			
			CoxIL2	GGCTACTGCCACTAATAATCG	C. Borgwardt, pers. comm.
			CoxIH2	CCTAAGAAGCCAATTGATATTATG	C. Borgwardt, pers. comm.
			COIf-ot1	TTGGTATAGRATTGGATCYCC	This study
			COIr-ot2	CGCCGGTACAGGATGAAC	This study
			COIf-ot2	CAGCAAGATGAAGGGAGAAGAT	This study
			COIr-ot1	CGAAACYTAAACACTACCTT	This study
cytb/CR	80	780			
			14943L	CCRTTYCACCCATACTTCTC	D. Ray, pers. comm.
			15789H	GGGTACATATTATCTTTYAMT	This study
LDH-A	57	735			
(intron)			LA17-F1	TGGCTGAAACTGTTATGAAGAACC	(Gatesy *et al.* 2004)
			LA17-R1	TGGATTCCCCAAAGTGTATCTG	(Gatesy *et al.* 2004)
RAG1	45	1776			
(exon)			RAGL1	ACTCGATTTTGTCACAATTG	(Gatesy *et al.* 2003)
			RAGR1	ATAGCTTCCAGCTCATCTGCTTG	(Gatesy *et al.* 2003)
			RAGL3	AAGGCTGTTTGCATGACTTTGTT	(Gatesy *et al.* 2003)
			RAGR5	AGCAAAGTTTCCATTCATCCTCAT	(Gatesy *et al.* 2003)
			RAG1int-F	AGCCACAAGGAGATGGAAGGGAAA	This study
			RAG1int-R	TGGTCCACATCCATGCTTCTCACT	This study

[a] number of sequences obtained for each gene fragment

For MP, I tested data partitions for congruence by using 100 replicates of the partition homogeneity tests (PHT, Farris *et al.* 1994), implemented in PAUP* 4.0b10 (Swofford 2002). MP analyses were conducted separately for mtDNA and nuDNA

fragments in MEGA 4.0 with unordered and equally weighted characters. Trees were constructed with heuristic (close-neighbor interchange) searches and branch swapping with 100random-addition sequence replicates. Gaps (insertion-deletions) were treated as a fifth character state. Node support was calculated using 500 non-parametric bootstrap replicates (Felsenstein 1985). A single combined data set was used for ML and BI tree construction. ML analysis was carried out in RAxML 7.0.0 (Stamatakis 2006) on the RAxML webserver (http://phylobench.vital-it.ch/raxml-bb). Ten starting trees were built with MP before ML optimization. Node support was provided with 100 rapid bootstrap replicates. I used the general time-reversible (GTR) substitution model with among-site variation modeled by the Γ distribution with four discrete rate categories (Lanave *et al.* 1984; Rodriguez *et al.* 1990; Yang 1994). One ML analysis of the concatenated data was performed with an unpartitioned alignment and then repeated with a partitioned alignment using all five loci, allowing for an independent GTR+Γ_4 model per partition. Branch lengths were optimized per partition. I used MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003; Altekar *et al.* 2004) to perform Bayesian inference of phylogeny, partitioning the alignment by locus and allowing for each partition to evolve under an independent GTR+ Γ_4 model. The analysis was run twice with four chains for 10 million Metropolis-coupled Markov chain Monte Carlo steps (MCMCMC, Huelsenbeck and Ronquist 2001), discarding the first 10% as burn-in. The heating parameter was fine-tuned to 0.02 so as to lead to better mixing. Convergence was assessed by examining the stationarity of the ln-likelihood and the effective sample size (ESS > 200) of each estimated parameter in Tracer 1.4 (http://tree.bio.ed.ac.uk/software/tracer), along with

the inspection of the standard deviation of split frequencies (<0.003) and successful state swap frequencies (in the range [0.25,0.85]). For all analyses, the Nile crocodile (*Crocodylus niloticus*) and slender-snouted crocodile (*Mecistops cataphractus*) were used as outgroup taxa.

Population Aggregation and Cladistic Haplotype Analyses

Because monophyly cannot be inferred accurately without demonstrating character fixation, a character-based assessment is required for distinguishing between phylogenetic species (Goldstein *et al.* 2000). This approach allows us to test the hypothesis of discrete terminal taxa first proposed by Schmidt (1919) between *Osteolaemus* and *Osteoblepharon* and also test a novel hypothesis of phylogeographic distinction among populations in the major drainages of Central Africa and those of West Africa. Population aggregation analysis (PAA, Davis and Nixon 1992) is a discrete character-based method that depends on alternate fixed characters to define phylogenetic species (sensu Cracraft 1983). PAA assumes fixed characters will be partitioned in geographic regions because related individuals are more likely to be associated spatially (Wright 1943). Beginning with an a priori hypothesis of possible allopatric speciation, I used PAA to scan each gene fragment for fixed and alternate character differences among three broad geographic regions sampled here - the Congo Basin (Republic of Congo and Demographic Republic of Congo), the Ogooué Basin (Gabon and Cameroon) and West Africa (Ghana and the Ivory Coast).

PAA can be used to assess sequence data from two perspectives, that of a gene representing a single attribute (i.e. haplotypes) or the sequence as a collection of linked but independent characters (nucleotides). Brower (1999) designated these two

methods as PAA1 and PAA2, respectively, and provided hypothetical and empirical examples showing how either approach can produce spurious results if used alone. I present a combined analysis using both single-attribute (PAA1) and linked-character (PAA2) methods. Aligned sequences were visually analyzed in MEGA for fixed, alternating nucleotide positions (characters) as well as for variable sites within geographic region (traits). PAA distances between regions are based on the percentage of fixed and alternate character states for each gene.

While advocating the combined use of both PAA1 and PAA2 approaches, Brower (1999) suggests a cladistic haplotype analysis (CHA) may be superior to either PAA method. PAA may ignore relevant information contained in sequence data because it disregards the hierarchical structure of the gene tree (PAA1) or dismisses important information by assuming all attributes shared between populations are homologous (PAA2), which may conflict with a more parsimonious interpretation of the data. CHA, on the other hand, evaluates the weight of evidence contained in homoplastic traits in order to estimate the phylogeny of haplotype groups. In this case, CHA is used to approximate phylogeny, group closely related organisms and test my a priori hypotheses of species boundaries (Brower 1999). I performed a CHA analysis using minimum spanning haplotype networks implemented in TCS 1.21 (Clement *et al.* 2000) to further test hypotheses of geographic separation of populations of dwarf crocodiles into diagnosable clades.

Morphology

No animals were killed for the purpose of this study. I salvaged three dwarf crocodile skulls from the wild in Gabon and acquired three skulls in Congo from

hunters. CITES and U.S. Fish and Wildlife permits allowed us to import only three skulls, which were accessioned to the American Museum of Natural History and referenced in this study – AMNH R160902 (*Mecistops cataphractus*, Rep. of Congo), AMNH R160900 (*O. t. osborni*, Rep. of Congo), AMNH R160901 (*O. t. tetraspis*, Gabon). I compared cranial features from these skulls and two from West Africa (AMNH R24740 and adult male from Liberia provided by J. Groves, N. Carolina Zoo) using morphological characters described by others (Schmidt 1919; Brochu 2007) with respect to geographical origin and my molecular data.

Additionally, I collected morphometric data from wild crocodiles in Gabon and from live crocodiles in bushmeat markets in Congo. Measurements included head length (HL, measured from tip of snout to medial posterior edge of the supraoccipital plate), total length (TL, noting whether tail was complete or damaged) and cranial table width (CT, supraoccipital plate measured lateromedially), as is standard in crocodilian research (Bayliss 1987). Nearly all measurements were taken by a single observer (MJE), reducing possible inter-observer error. To control for crocodile age and total body length, I compared the ratio of head length to total length between regions. Because head length increases isometrically with body length, it is often used to estimate total length (Greer 1974; Woodward *et al.* 1995); total length relative to head length may also serve as a descriptor of body shape evolution in the context of life history (habitat use, etc.). I additionally compared a measure of cranial proportion, head width to head length (HL/CT), between animals from Congo and Gabon. Hall (1989) used similar head shape proportions to compare geographically isolated populations of New-Guinea crocodiles (*Crocodylus novaeguineae*) and to