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FROM THE COVER

An ancient icon reveals new mysteries: mummy DNA resurrects a cryptic species within the Nile crocodile

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Abstract

The Nile crocodile (*Crocodylus niloticus*) is an ancient icon of both cultural and scientific interest. The species is emblematic of the great civilizations of the Nile River valley and serves as a model for international wildlife conservation. Despite its familiarity, a centuries-long dispute over the taxonomic status of the Nile crocodile remains unresolved. This dispute not only confounds our understanding of the origins and biogeography of the 'true crocodiles' of the crown genus *Crocodylus*, but also complicates conservation and management of this commercially valuable species. We have taken a total evidence approach involving phylogenetic analysis of mitochondrial and nuclear markers, as well as karyotype analysis of chromosome number and structure, to assess the monophyletic status of the Nile crocodile. Samples were collected from throughout Africa, covering all major bioregions. We also utilized specimens from museum collections, including mummified crocodiles from the ancient Egyptian temples at Thebes and the Grottes de Samoun, to reconstruct the genetic profiles of extirpated populations. Our analyses reveal a cryptic evolutionary lineage within the Nile crocodile that elucidates the biogeographic history of the genus and clarifies long-standing arguments over the species' taxonomic identity and conservation status. An examination of crocodile mummy haplotypes indicates that the cryptic lineage corresponds to an earlier description of *C. suchus* and suggests that both African *Crocodylus* lineages historically inhabited the Nile River. Recent survey efforts indicate that *C. suchus* is declining or extirpated throughout much of its distribution. Without proper recognition of this cryptic species, current sustainable use-based management policies for the Nile crocodile may do more harm than good.

Keywords: ancient DNA, African biogeography, *Crocodylus*, *C. niloticus*, *C. suchus*, mummy

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Introduction

The idea that taxonomy is destiny (May 1990) is particularly relevant to the conservation and management of crocodilians (Hutton 2000). Current policies intended to promote sustainable harvest of managed crocodile populations are based predominantly on morphological criteria that provide limited taxonomic and phylogenetic

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We dedicate this work to our co-author, John Thorbjarnarson, who passed during the final preparation of this manuscript and whose unwavering commitment to crocodile conservation has been an inspiration to all of us.

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resolution (Brazaitis 1973; Ross 1998). Assumptions of genetic homogeneity and continuing taxonomic uncertainty within this group raise the concern that management plans may not adequately protect extant diversity and evolutionary potential, especially in more widespread species. This situation is exemplified by the Nile crocodile (*Crocodylus niloticus*), a widespread, commercially exploited species that has become a model of international wildlife conservation (Ross 1998; Hutton 2000; Fergusson 2010) despite a history of taxonomic discord that has persisted since the eighteenth century (Table 1; Fuchs *et al.* 1974, King & Burke 1989).

The Nile crocodile is comprised of 11 synonymized, historically described species and seven previously proposed subspecies (Table 1). As currently managed, the species is recognized as a single entity, although recent molecular studies provide evidence to the contrary. Limited phylogenetic studies indicate that *C. niloticus* is paraphyletic (Schmitz *et al.* 2003; Meredith *et al.* 2011), and multilocus microsatellite comparisons have shown that populations across Africa are geographically differentiated (Hekkala *et al.* 2009).

Although the Nile crocodile is considered widespread with a largely sub-Saharan distribution, managing this culturally and commercially valuable species as a single, widespread evolutionary lineage may be contributing to a globally significant loss of crocodilian diversity (Hekkala *et al.* 2009; Shirley *et al.* 2009). This concern is particularly important in western regions with populations that are increasingly susceptible to range contraction and local extirpation (Shirley *et al.* 2009). For example, populations were found in the central Sahara until the late nineteenth century (de Smet 1999) though only small isolates may persist in some locales today (Shine *et al.* 2001).

Here we test the hypothesis that the Nile crocodile is a single, homogeneous evolutionary lineage through total evidence molecular analysis of 5016 bp of mitochondrial and nuclear sequence data from samples collected from wild populations across Africa and Madagascar (Fig. 1, Table 2). We provide a complementary temporal perspective spanning over 2 200 years through diagnostic haplotype analysis of historical specimens from museum holdings, including crocodile mummies from the ancient Egyptian sites of Thebes and the Grottes de Samoun. Finally, we compare our sequence-based conclusions with karyotype analysis.

Methods

Contemporary samples and markers

We collected 123 samples of Nile crocodiles from throughout Africa (Fig. 1, Table 2). Collections were made from wild or wild-caught, ranch-held individuals and consisted of tail tissue or fresh blood (<0.5 mL) either in lysis buffer or dried on Whatman filter paper.

Table 1 Taxonomic History of the Nile Crocodile. Locality refers to the type locality designation in the literature description, which may not be the same as the origin of the type specimen for that taxon

Taxon	Author and Year	Locality
Crocodylus niloticus	Laurenti 1768	Egypt
Synonyms		
Crocodylus vulgaris	Cüvier 1807	Egypt
Crocodylus suchus	Geoffroy Saint-Hilaire 1807	Nile and Niger Rivers
Crocodilus multiscutatus	RÜPPELL in Cretzschmar 1826	Sudan
Crocodilus marginatus	GEOFFROY 1827	Egypt
Crocodilus lacunosus	GEOFFROY 1827	Egypt
Crocodilus complanatus	GEOFFROY 1827	Egypt
Crocodilus octophractus	RÜPPELL in GRAY in Griffith & Pidgeon 1831	Sudan
Alligator cowieii	SMITH in Hewitt 1937	South Africa
Crocodylus binuensis	Baikie 1857	Nigeria
Crocodilus madagascariensis	Grandidier 1872	Madagascar
Crocodilus vulgaris var. madagascariensis	Boettger 1877	Madagascar
Crocodilus hexaphractos	RÜPPELL in SCHMIDT 1886 (*nomen nudum*)	Sudan
Proposed subspecies		
Crocodylus niloticus niloticus	LAURENTI 1768	Egypt
Crocodylus niloticus africanus	LAURENTI 1768	East Africa
Crocodylus niloticus chamses	Bory de Saint-Vincent 1824	Southern Congo
Crocodylus niloticus cowiei	SMITH in Hewitt 1937	South Africa
Crocodylus niloticus madagascariensis	Grandidier 1872	Madagascar
Crocodylus niloticus pauciscutatus	Deraniyagala 1948	Kenya
Crocodylus niloticus suchus	Geoffroy Saint-Hilaire 1807	West Africa

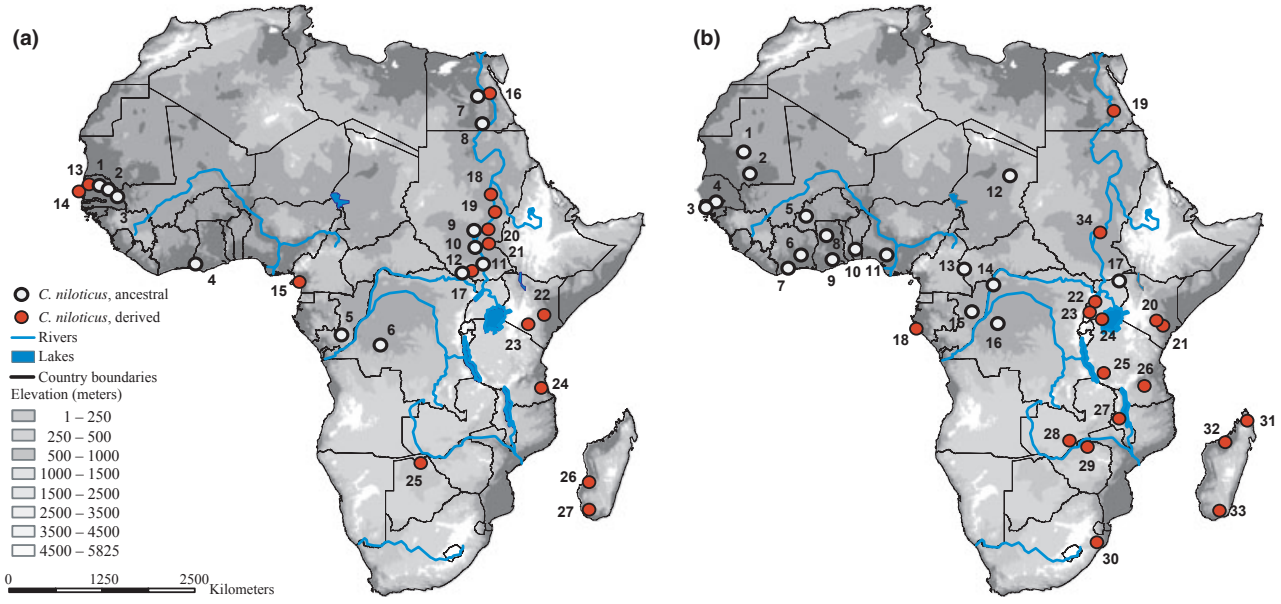

Fig. 1 Map of sample localities showing the distribution of ancestral (*white*) and derived (*red*) haplotypes for historical pre-1975 (a) and contemporary post-1975 (b) specimens.

To better understand the evolutionary history of *C. niloticus* in relation to true crocodiles, our analyses included data from samples of seven other *Crocodylus* species representing both Asian and New World lineages. The remaining members of the Crocodylinae (*Osteolaemus tetraspis* and *Mecistops cataphractus*) and *Alligator mississippiensis* served as outgroups, reflecting the most recent phylogenetic hypotheses for the crown group of the Crocodylidae and the Order Crocodylia (Gatesy & Amato 1992; Brochu 2003; McAliley *et al.* 2006; Meredith *et al.* 2011). These taxa were included from samples taken from captive specimens (St. Augustine Alligator Farm, St. Augustine, FL, USA) or previously published sequences available on Genbank as follows: *C. rhombifer*, *C. acutus*, *C. moreletii*, *Mecistops cataphractus* and *Osteolaemus tetraspis* (all amplified and sequenced as part of this study), *C. intermedius* (12s—AY239132, 16s—AY239146, dloop—AF460207, rag1—AY239173), *C. porosus* (12s—AY770534, 16s—EU621805, dloop—AF460213, WANCY—DQ273698, ND4—AJ810453), *C. siamensis* (mtDNA—EF581859, rag1—AY136677) (Ray & Densmore 2002; Gatesy *et al.* 2003).

We examined sequence variation across a total of 5 016 bp from nine gene regions. Five regions (2761 bp) were mitochondrial (mtDNA) and four were nuclear (nDNA) (2254 bp), as follows: control region/dloop (735 bp); 12s rRNA (421 bp); 16s rRNA (415 bp); WANCY tRNA cluster (Seutin *et al.* 1994) from the ND2-flanking region including tRNA_Trp, tRNA_Ala, tRNA_Asn, tRNA_Cys, and part of tRNA_Tyr (330 bp); NADH dehydrogenase 4 (ND4, 860 bp); recombination-activating gene 1 (rag1, 469 bp); ribosomal protein S6 (693 bp); and introns for tropomyosin (330 bp) and ornithine decarboxylase (762 bp) (Friesen *et al.* 1999).

Contemporary sample data collection

DNA was extracted using Qiagen Easy-DNA extraction kits or standard phenol–chloroform methods. Extraction products were stored at 50 ng/μL. PCR cocktails and cycling conditions were optimized for each marker (Table S1, Supporting information) and amplifications were performed on an ABI 9700 thermocycler in 20–25 μL volumes. Sanger sequencing reactions were carried out using BigDye v3.1 sequencing kits in 6–8 μL volumes. Gene regions were sequenced in both directions on either an ABI 3700 or 3730XL automated capillary sequencer. Base calling was performed with Sequencher v4.1 (Genecodes Corp.). Consensus sequences were produced with CLC v3.6.2. Marker datasets were compiled and aligned individually in MEGA4 (Tamura *et al.* 2007) utilizing Clustal W (Larkin *et al.* 2007) (Gap penalties = 50, Gap Extension penalties = 25) and checked by eye prior to concatenation.

Contemporary sample analyses

Sequence data were first analyzed for fixed characters using Population Aggregation Analysis (Davis & Nixon 1992) and terminal taxa with unique and fixed characters were subsequently examined for phylogenetic structure with data from all species combined by genome and concatenated for total evidence analysis (Maddison 1997;

Table 2 Contemporary and Historical Samples Utilized in This Study. Locality and sampling data for each specimen utilized in this study. For archival material, both the original collection locality and the museum accession information are listed. Terminal Label refers to the specimen ID given in Fig. 2, Figs S1 and S2, Table 3, and Table S3. Museum acronyms: AMNH—American Museum of Natural History (New York, NY, USA), CAS—California Academy of Sciences (San Francisco, CA, USA), FLMNH—Florida Museum of Natural History (Gainesville, FL, USA), MNHN—Museum Nationale d'Histoire Naturelle (Paris, France), USNM—National Museum of Natural History, Smithsonian Institution, Washington, DC, ZFKM—Alexander Koenig Zoological Research Museum, Bonn, Germany). Genbank accession numbers are listed next to individuals from Schmitz *et al.* 2003. * Indicates only short 12s and/or dloop fragments were sequenced

Map Number	Terminal Label	Country	Locality	Collector	Year Collected	Museum	Specimen#	Notes
Figure 1A*								
1	SENEGAL_1825	Senegal	UNK	G.S. Perrottet & F.M.R. Leprieur	1825–1829	MNHN	1977_1606	
2	SENEGAL_1934	Senegal	Kedougou(a)	F.C. Wonder	1934	FMNH	20798	
3	SENEGAL_1824	Senegal	UNK	Brongniart	approx. 1824	MNHN	2175	
4	IVORY COAST_1885	Cote-d'Ivoire	Assinie	Chaper	1885	MNHN	1885407	
5	REP CONGO_1882	Republic of Congo	N'ganchou	P.S. de Brazza	1886	MNHN	1886_182	Not included in analysis, partial sequence identical to REP CONGO_1886
5	REP CONGO_1886	Republic of Congo	N'gouchou	P.S. de Brazza	1882	MNHN	1886_186	
6	DEM REP CONGO_1924	Dem. Republic of Congo	Kasai River	Father R. Callewaert	1924	AMNH	28904	
7	MUMMY_THEBES_A	Egypt	Mummy - Grottes de Thebes	Cailloud	700–2200 YBP	MNHN	1986_1471	brought from Egypt 1820's
7	MUMMY_THEBES_B	Egypt	Mummy - Grottes de Thebes	Cailloud	700–2200 YBP	MNHN	1986_1473	brought from Egypt 1820's
7	MUMMY_THEBES_C	Egypt	Mummy - Grottes de Thebes	Cailloud	700–2200 YBP	MNHN	1986_1479	brought from Egypt 1820's
7	MUMMY_HAUTE	Egypt	Mummy, Haute Egypt	V. Schoelcher	700–2200 YBP	MNHN	1886_445	
8	MUMMY_SAMOUN_A	Egypt	Mummy - Grottes de Samoun	Gervais	700–2200 YBP	MNHN	1986_1475	
8	MUMMY_SAMOUN_B	Egypt	Mummy - Grottes de Samoun	Gervais	700–2200 YBP	MNHN	1986_1478	
8	MUMMY_SAMOUN_C	Egypt	Mummy - Grottes de Samoun	Pariset	700–2200 YBP	MNHN	1986_1480	
9	SUDAN_MELUT_1922	Sudan	Melut	Anthony	1927	AMNH	42962	
10	SUDAN_WNA_1922	Sudan	White Nile	Taylor	1922	AMNH	23464	
11	SUDAN_WNB_1922	Sudan	White Nile	Taylor	1922	AMNH	23465	
12	ZIMBABWE_1911	Zimbabwe	Faradje	Lang - Chapin Expedition	1911	AMNH	10079	
13	SENEGAL_1803	Senegal	UNK	C. Heudelot	1803–1827	MNHN	7364	
14	SENEGAL_1768	Senegal	UNK	Adanson	1749–1754	MNHN	7524	*Crocodylus vert* TYPE
15	CAMEROON_1966	Cameroon	Edea, Sanaga River	T.J. Papenfuss	1966	CAS	133814	
16	EGYPT_1822	Egypt	Nile	T. Duvant	approx. 1822	MNHN	7546	*Crocodylus vulgaris* PARATYPE

Table 2 (*Continued*)

Map Number	Terminal Label	Country	Locality	Collector	Year Collected	Museum	Specimen#	Notes
17	ZIMBABWE_1912	Zimbabwe	Faradje	Lang - Chapin Expedition	1912	AMNH	10081	
18	SUDAN_UN_1922	Sudan	Zeraf, Upper Nile	Taylor	1922	AMNH	23471	
19	SUDAN_WNC_1922	Sudan	White Nile	Taylor	1922	AMNH	23466	
20	SUDAN_WND_1922	Sudan	White Nile	Taylor	1922	AMNH	23469	
21	SUDAN_WNE_1922	Sudan	White Nile	Taylor	1922	AMNH	23470	
22	KENYA_1960	Kenya	Garissa	R.H. Pine	1960	AMNH	88634	
23	KENYA_1919	Kenya	Nairobi	H.C. Raven	1919–1920	USNM	63592	
24	TANZANIA_1972	Tanzania	UNK	USFWS Confiscation	1972	AMNH	108941	
25	BOTSWANA_1967	Botswana	Shakawe	T. Liversedge	1967	USNM	195448	
26	MADAGASCAR_1885	Madagascar	Tulear	A. Grandidier	1870	MNHN	6498	*Crocodylus madagascariensis* TYPE
27	MADAGASCAR_A_1931	Madagascar	Amboasary	H. Bluntschli	1931	AMNH	71192	
27	MADAGASCAR_B_1931	Madagascar	Amboasary	H. Bluntschli	1931	AMNH	142496	
27	MADAGASCAR_C_1931	Madagascar	Amboasary	H. Bluntschli	1931	AMNH	71191	
Figure 1B								
1	MAURITANIA_1	Mauritania	Matmata	S. Robin	1993	MNHN	1993_5805*	Short 12S and dloop sequences only
2	MAURITANIA_2	Mauritania	Aioun el-Atrouss	Bohme	UNK	ZFMK	Uncatalogued	From 4 specimens utilized in Schmitz *et al.* 2003
3	SENEGAL	Senegal	Casamance River	M.H. Shirley	2008	FLMNH	Uncatalogued	Djibelor Crocodile Farm - wild stock
4	GAMBIA_1	The Gambia	Kedougou, Gambia River	W. Bohme	UNK	N/A		Specimen utilized in Schmitz *et al.* 2003
4	GAMBIA_2	The Gambia	River Gambia NP	M.H. Shirley	2008	FLMNH	Uncatalogued	
4	GAMBIA_3	The Gambia	River Gambia NP	M.H. Shirley	2008	FLMNH	Uncatalogued	
5	BURKINA FASO	Burkina Faso	UNK	Bohme	UNK	N/A		
6	IVORY COAST_1	Cote-d'Ivoire	Abi Lagoon	M.H. Shirley	2006	FLMNH	Uncatalogued	Not included in phylogenetic analysis, same as haplotype found at Site 7
7	IVORY COAST_2	Cote-d'Ivoire	Go River	M.H. Shirley	2006	FLMNH	Uncatalogued	
8	GHANA_1	Ghana	Mole National Park	M.H. Shirley	2006	FLMNH	Uncatalogued	
9	GHANA_2	Ghana	Legon Farms Dam, Accra	M.H. Shirley	2006	FLMNH	Uncatalogued	Not included in phylogenetic analysis, same as haplotype found at Site 8
10	BENIN*	Benin	UNK	R. Bourgat	1978	MNHN	1978_2051	
11	NIGERIA	Nigeria	Escravos River, Niger Delta	M.P.O. Dore	2009	FLMNH	Uncatalogued	Bushmeat sample collected in Benin City
12	CHAD	Chad	Ennedi	M. Klemens	1997	AMNH	145361*	Short 12S and dloop sequences only

Table 2 (*Continued*)

Map Number	Terminal Label	Country	Locality	Collector	Year Collected	Museum	Specimen#	Notes
13	CENTRAL AFR REP	Central African Republic	Berberati, near Bangui	L. Chirio	1995	MNHN	1997_3171*	MNHN 1997_3171, Short 12S and dloop sequences only
14	REP CONGO_1*	Republic of Congo	Dougou, Oubangi River	V. de Buffrenil	1986	MNHN	1987_1120	
14	REP CONGO_2*	Republic of Congo	Likouala (Edzala?)	V. de Buffrenil	1986	MNHN	1987_1114	
14	REP CONGO_3*	Republic of Congo	Lukouala, Congo	V. de Buffrenil	1986	MNHN	1986_1945	
15	REP CONGO_4	Republic of Congo	Likouala aux Herbes	M.J. Eaton	2004	FLMNH	Uncatalogued	
16	DEM REP CONGO	Dem. Republic of Congo	Lac Mai Ndombe	R. Fergusson	2002	N/A		Bushmeat sample collected in Inongo
17	UGANDA_1	Uganda	Kidepo Valley NP	M.H. Shirley	2009	FLMNH	Uncatalogued	
17	UGANDA_2	Uganda	Kidepo Valley NP	M.H. Shirley	2009	FLMNH	Uncatalogued	
18	GABON_1	Gabon	Petit Loango, Loango NP	M.J. Eaton	2006	FLMNH	Uncatalogued	
18	GABON_2	Gabon	Petit Loango, Loango NP	M.J. Eaton	2006	FLMNH	Uncatalogued	
19	EGYPT_1	Egypt	Lake Nasser, near Aswan	M.H. Shirley	2008	FLMNH	Uncatalogued	
19	EGYPT_2	Egypt	Lake Nasser, near Aswan	M.H. Shirley	2008	FLMNH	Uncatalogued	
19	EGYPT_3	Egypt	Lake Nasser, near Aswan	M.H. Shirley	2008	FLMNH	Uncatalogued	
19	EGYPT_4	Egypt	Lake Nasser, near Aswan	M.H. Shirley	2008	FLMNH	Uncatalogued	
19	EGYPT_5	Egypt	Lake Nasser	UNK	UNK	ZFMK	Uncatalogued	12s sequence from Schmitz *et al.* 2003 (AY195943)
20	KENYA_1	Kenya	Tana River	R. Fergusson	2001	N/A		
21	KENYA_2	Kenya	Tana River	R. Fergusson	2001	N/A		
21	KENYA_3	Kenya	Tana River	R. Fergusson	2001	N/A		
22	UGANDA_3	Uganda	Victoria Nile, Murchison Falls NP	M.H. Shirley	2010	FLMNH	Uncatalogued	
22	UGANDA_4	Uganda	Semliki River, Semuliki NP	M.H. Shirley	2010	FLMNH	Uncatalogued	
23	UGANDA_5	Uganda	Lake Edward, Queen Elizabeth NP	M.H. Shirley	2010	FLMNH	Uncatalogued	
24	UGANDA_6	Uganda	Lake Mburo, Ruizi Drainage, Lake Mburo NP	M.H. Shirley	2010	FLMNH	Uncatalogued	
25	TANZANIA_2	Tanzania	Lake Rukwa	R. Fergusson	2001	N/A		
26	TANZANIA_1	Tanzania	Rufiji River	R. Fergusson	2001	N/A		
27	MALAWI	Malawi	Salima Bay	R. Fergusson	2001	N/A		
28	ZIMBABWE_1	Zimbabwe	Lake Kariba	UNK	UNK	N/A		12s sequence from Schmitz *et al.* 2003 (AY 195954/55)
28	ZIMBABWE_2	Zimbabwe	Lake Kariba	UNK	UNK	N/A		12s sequence from Schmitz *et al.* 2003 (AY 195954/55)
29	ZIMBABWE_3	Zimbabwe	Lake Kariba	R. Fergusson	2002	N/A		
30	SOUTH AFRICA	South Africa	Lake St. Lucia	A. Leslie	UNK	N/A		
31	MADAGASCAR_1	Madagascar	Ankarana Caves	Garcia	2002	N/A		
31	MADAGASCAR_2	Madagascar	Ankarana Caves	Garcia	2002	N/A		
32	MADAGASCAR_3	Madagascar	Betsiboka River	E. Hekkala	2000	N/A		
33	MADAGASCAR_4	Madagascar	Estuary, Fort Dauphin	de Huelme	2002	N/A		
34	SUDAN	Sudan	Chor Melk en-Nasir	UNK	UNK	ZFMK	50489	12s sequence from Schmitz *et al.* 2003 (AY195953)

Kluge 1998). Prior to analysis, individual marker datasets were tested for the maximum likelihood model of evolution with jModelTest 0.1.1 (Posada 2008) and MrModelTest2.3 (Nylander *et al.* 2004) for a *C. niloticus*-only dataset and a dataset including *Crocodylus* outgroups. Where the inferred model of evolution was not consistent between datasets, we chose the model selected for the *C. niloticus*-only data. Datasets were tested for congruence and analyzed in PhyML (Guindon & Gascuel 2003) and MrBayes (Ronquist & Huelsenbeck 2003) to generate hypotheses of phylogenetic structure under maximum likelihood and Bayesian algorithms as follows:

Maximum likelihood. A PhyML search was implemented on the Montpellier Bioinformatics Platform (http://www.atgc-montpellier.fr/phyml). The full, concatenated dataset was analyzed under HKY85+I+G substitution model as per the recommendation of jModelTest 0.1.1. Trees were searched from a starting tree created by BIONJ using the best of the SPR and NNI options with topologies and branch lengths optimized. Branch support was determined with both the SH-Like and Chi^2-based options of the Approximate Likelihood Ratio Test (aLRT) method (Anisimova & Gascuel 2006), as well as nonparametric bootstrapping over 100 replicates. To test the hypothesis of *C. niloticus* monophyly, we compared the resulting topology to a constrained tree compiled in MacClade4.01 (Maddison 1997) wherein *C. niloticus* represented a monophyletic group. Additional ML searches were conducted and the likelihood values for the constrained and unconstrained topologies were compared using the Shimoduro–Hasegawa option in PAUP4.0b10 (Swofford 2002). Statistical measures for rejection of the hypothesis of no difference were set at 95%.

Bayesian inference. The concatenated dataset was partitioned by gene region with the substitution model implemented for each gene (12s—HKY+I, 16s—GTR+G, dloop—HKY+I, ND4—GTR+G, WANCY—HKY, rag1—JC, OD—F81, TROP—F81, S6—F81, mtDNA—HKY+I+G, nDNA—HKY+I) where all model parameters were estimated by MrModelTest2.3 (Nylander *et al.* 2004). Gaps (indels) were coded as restriction site binary characters. Three simultaneous Markov Chain Monte Carlo searches were run with five chains for 12 000 000 generations with trees sampled every 500 generations. A 50% majority rule consensus tree was created after discarding the first 2000 'burn-in' trees. Trees were rooted by both outgroup and mid-point rooting methods; both methods produced the same root point (Hess *et al.* 2007).

We used BEAST v1.5beta2 (Drummond *et al.* 2006), which implements a Bayesian MCMC method and a relaxed molecular clock approach (Drummond 2007), to estimate divergence times. We assumed a relaxed lognormal model of lineage variation and a Yule prior for branching rates. We examined rates using the combined dataset (nuDNA and mtDNA) partitioned by gene region, as well as by coding versus non-coding regions. The coding regions were further partitioned according to 1 + 2 and 3 codon positions and the substitution model, rate heterogeneity and base frequencies were unlinked across codon positions [(1 + 2), 3].

For calibration, we used fossil record-based estimates of the divergence between *Alligator* and *Crocodylus* (ca. 79 mya), *Crocodylus* and *Mecistops/Osteolaemus* (at ca. 20–24 mya), as well as the earliest fossil appearances of *C. niloticus* in Africa (ca. 3–7 mya) (Brochu 2004c; Brochu personal communication), and *Crocodylus* in the Caribbean (conservatively estimated at 4–5 mya; Miller 1980). We used these dates as lognormal distribution priors for each respective node setting the offset as the minimum age (A. Drummond personal communication). We placed monophyly constraints on the New World clade and on eastern *C. niloticus*, respectively, thus attaining the same general topology as assessed by the full phylogenetic analyses. Three replicates were run for 100 000 000 generations each with tree and parameter sampling occurring every 1000 generations. The adequacy of a 10% burn in and convergence of all parameters were assessed using the software TRACER v1.4.1 (Rambaut & Drummond 2005). The sampling distributions of the three independent replicates were then combined using the software LogCombiner v1.5 and the resulting 360 000 000 samples summarized and visualized using the software Tree Annotator v1.5 and FigTree v1.2 (Rambaut 2006).

Mean intra- and inter-clade distances (i.e. number of base substitutions per site from averaging over all sequence pairs within and between groups) were calculated in MEGA4 for both the combined and the mtDNA only datasets (Tamura *et al.* 2007). Sequences for captive individuals were removed from all analyses, and divergence estimates for pairs not including *Alligator* were estimated with the preceding datasets minus *Alligator*. Analyses were conducted using Maximum Composite Likelihood (Tamura *et al.* 2004). The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons (Tamura & Kumar 2002). Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. Standard error estimates were obtained by bootstrapping over 500 replicates.

Ancient DNA methods

Tissue was harvested from 57 dried or ethanol preserved museum specimens from eight institutions, including both natural history and anthroplogical collections (Table 2). We sampled Egyptian crocodile mummies from the Phoebe Hearst Museum (PHM) at the University of California, Berkley; the University of Pennsylvania Museum of Anthroplogy (UPenn); the British Museum (BM); and the Musée National d'Histoire Naturelle (MNHN) (Table S3, Supporting information). During all archival tissue collections, surgical utensils were sterilized and work areas were wiped with DNAaway (Molecular Bioproducts) between samples. Specimen surfaces were wiped with 20% Clorox bleach and air dried prior to sampling.

Mummified crocodile hatchlings from MNHN, PHM and UPenn were very fragile and handled separately. Individuals from MNHN were originally collected from two sealed tombs (Grotte de Samoun and Grotte de Thebes) in the early 1800s and are estimated to have been interred between 200 BC and 200 AD (S. Ikram, Cairo Museum, personal communication.). One hatchling from PHM was from collections noted as 'predynastic' Egypt (estimated ≥3100 BC), while one from Upenn was undated. For each hatchling a cross section of the tail, including bone and muscle tissue, was sampled, rinsed with 20% Clorox bleach and sterile water prior to hydration in glycine buffer for 1 week to 3 months with regular fluid changes (Shedlock *et al.* 1997). Samples from adult mummies and more recent specimens (nineteenth and twentieth centuries), were soaked for 36–76 h in PBS with multiple fluid changes.

All museum samples were processed in clean room facilities, separate from contemporary samples. Processing of each specimen was replicated in at least one additional institution [either American Museum of Natural History aDNA Laboratory (AMNH), University of Nevada Reno (UNR), U.S. EPA aDNA Laboratory, Cincinnati, OH (EPA), or Tulane University (TU)]. At each institution DNA extraction, PCR setup and post-PCR handling of archival samples took place in physically separate locations with procedures following precautionary protocols recommended for use with degraded or ancient DNA (Cooper & Poinar 2000; Paabo *et al.* 2004; Gilbert *et al.* 2005; Willerslev & Cooper 2005). Facilities at AMNH and EPA were equipped with positive air pressure, wall mounted UV lamps, protective disposable lab attire, and direct shipping of all equipment and reagents, while those at TU and UNR consisted of separate, dedicated lab space.

DNA extraction from archival museum specimens consisted of a modified Qiagen DNeasy tissue protocol after extended hydration in either PBS or Glycine buffer. All samples were handled in batches of 6 with the exception of mummies, which were processed as batches 'per institution' of 4–8 samples. Negative controls were included throughout the process for each batch of samples. During tissue digestion, 5 μL of 1 M dithiothreitol (DTT) was added along with proteinase K to enhance protein digestion. Care was taken to mix reagents by hand at each step rather than risk shearing the DNA by vortexing. Samples were eluted in two separate volumes of 75 μL with elution buffer warmed to 56 °C after resting in the column for 15 min.

All pre- and post PCR handling was physically separated, and involved use of both positive and negative controls. Positive PCR controls were added after archival tubes were sealed and placed on the thermocycler. Primers were designed from modern crocodile sequences to amplify ±187–200 bp each of mitochondrial 12s rRNA and d-loop gene regions covering previously identified hypervariable sites (12s183 5'TTGCCCT AAGCAGCCTGTAT3', 12s375 5'CCGTCTTTGACAGTC CTGGT3'; and ncdlpFs 5'GCCGACATTCTTATTAAAC-TAC3', ncdlpRs 5'GCAGATAAATGAATGCCTTAT3', Table S1). In addition, we attempted to amplify a 600 bp gene region using crocodile specific 12s primers to confirm that no contemporary DNA was present in aDNA extracts (Paabo *et al.* 2004).

Template DNA was amplified using GE Illustra puretaq PCR beads in 25 μL volumes and amplification products were visualized on a 1% agarose gel with EtBr staining. Successfully amplified PCR products were cleaned using ExoSAP-IT® (Affymetrix). Sanger sequencing reactions were carried out using BigDye v3.1 sequencing kits in 6–8 μL volumes. Gene regions were sequenced in both directions on either an ABI 3100, 3700 or 3730XL automated capillary sequencer. Base calling was performed with Sequencher v4.1 (Genecodes Corp.). In case of sequence ambiguity, archival tissue samples were re-extracted, amplified and sequenced up to three times for verification (Paabo *et al.* 2004).

Historical specimen sequence analyses

Both 12s and d-loop sequences from archival specimens were individually aligned with sequences from contemporary specimens. Assignment of each archival specimen to an evolutionary lineage was based on diagnostic characters found in sequences from contemporary specimens. Nucleotide sites were considered diagnostic if they were variable with fixed base differences between clades. We utilized a PAA (Davis & Nixon 1992) approach to assign historical specimens to clades with the program CAOS (Character Attribute Organization System; Sarkar *et al.* 2009).

As an exploratory measure, we performed a phylogenetic analysis of the aligned short fragment sequence data using a maximum likelihood approach as implemented in PhyML with the substitution model implemented HKY+I, as previously estimated by jModelTest 0.1.1 (Posada 2008).

Karyotyping

Samples for karyotype analysis were collected from Nile crocodiles at the St. Augustine Alligator Farm Zoological Park and had the following accession numbers: SAAF_1—93220, SAAF_edpool—A01026, and SAAF_2—93044. Karyotyping was conducted on four cell lines. Skin biopsies were taken from the toe webbing of captive individuals and primary fibroblast cell lines were established and preserved in the San Diego Zoo's Frozen Zoo® cell repository. Harvests and chromosome banding followed Kumamoto *et al.* (1996) with the exception of a 33 °C cell culture incubation temperature. We also obtained DNA sequence data from these individuals, following the protocols for contemporary specimens presented above, for comparison to natural populations and to address concerns about potential hybridization in captivity.

Results

All phylogenetic methods used to examine our combined mtDNA and nDNA sequence dataset recovered a paraphyletic *C. niloticus*, with a predominantly western African clade sister to a monophyletic clade comprised of a predominantly eastern African *C. niloticus* plus the four New World *Crocodylus* species (Fig. 2). Tree topologies with significantly weaker support values were recovered when *C. niloticus* monophyly was imposed. Mean, corrected sequence divergence estimates showed little intraclade divergence (<0.3%) for both the total, concatenated dataset and the mtDNA dataset in both *C. niloticus* clades (Table S2, Supporting information). Mean intraclade divergence estimates between the eastern and western clades did not overlap with mean

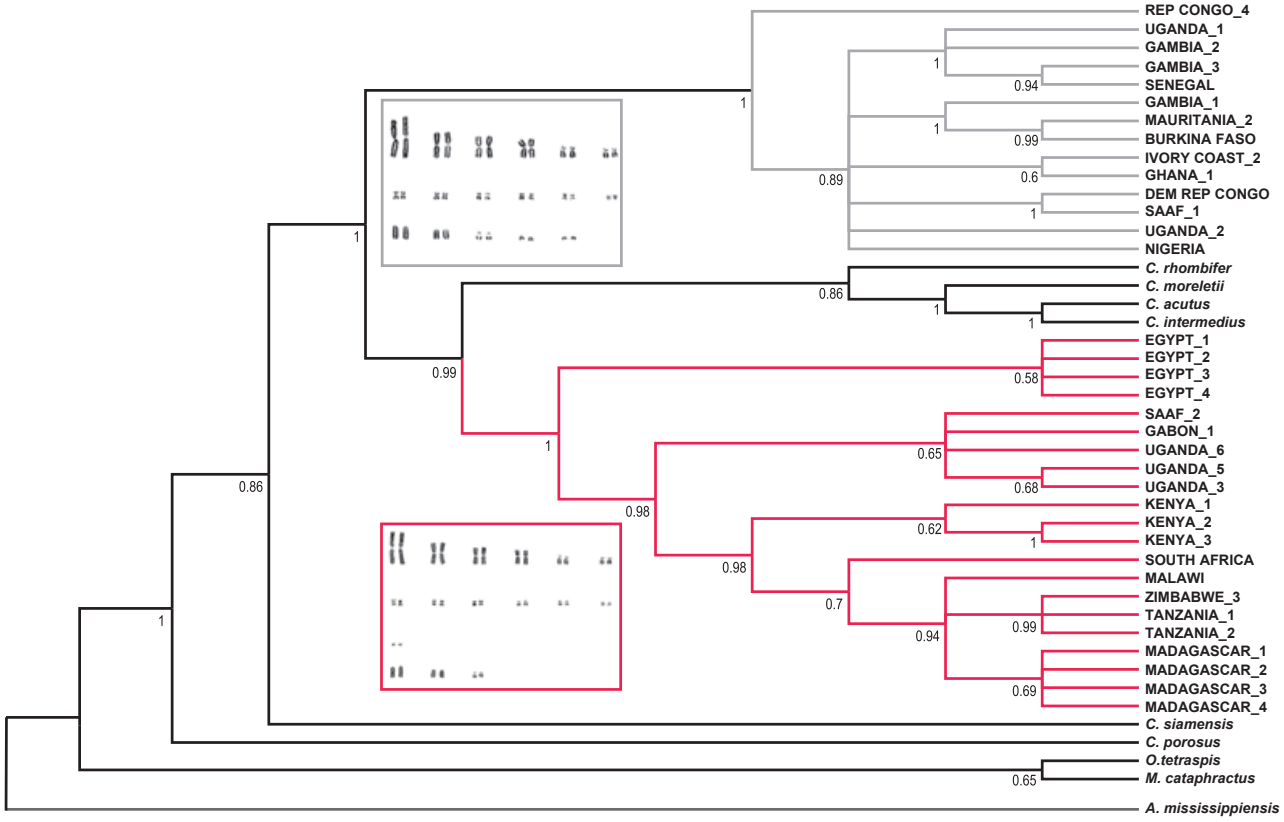

Fig. 2 Phylogenetic tree illustrating results of the Bayesian analysis of the full dataset, with karyotype insets. As illustrated, both the phylogenetic and karyotype analyses support a paraphyletic *C. niloticus* with the predominantly western clade (light grey) as sister to a monophyletic New World and Eastern *C. niloticus* clade. Posterior Probabilities (PP) are indicated above branches. Significant support is indicated by PP > 0.90. Individuals SAAF_1, SAAF_P (western) and SAAF_2 (eastern) exhibit the karyotypes displayed in the insets. Both BY and ML analyses resulted in similar tree topologies.

interclade divergence values, which were more than an order of magnitude higher (>4%), for both the total concatenated dataset and the mtDNA dataset (Table S2).

Karyotyping of representative captive individuals from each clade affirmed sequence-based evidence of evolutionary divergence between the two *C. niloticus* lineages (Fig. 2, inset). Consistent with prior findings, the derived eastern *C. niloticus* clade exhibits 32 chromosomes, comprised of 26 metacentric-submetacentric and six acrocentric elements. The ancestral western *C. niloticus* clade exhibits 34 chromosomes consisting of 24 metacentric–submetacentric and 10 acrocentric elements.

Divergence time estimates from the BEAST analyses of the full dataset partitioned by gene and partitioned by coding region and codon position were similar (i.e. the mean estimated dates from one analysis fell within the 95% confidence intervals of the other analysis), though mean ages were generally older and the confidence intervals were larger when the data were partitioned by gene region. Hence, we report only the outcome of the analysis based on coding region and codon position. Divergence time estimates suggest that the western *C. niloticus* lineage last shared a common ancestor with the New World-Eastern *C. niloticus* clade approximately 8.13 mya (5.24–12.64 mya, 95% CI tmrca) (Fig. S1, Supporting information). The western clade was estimated to have arisen ca. 2.455 mya (0.903–4.722 mya, 95% CI tmrca) (Fig. S1). The eastern *C. niloticus* lineage was estimated to have last shared a common ancestor with the New World clade approximately 5.7 mya (3.69–8.44 mya, 95% CI tmrca) (Fig. S1).

We sequenced up to 197 bp of the 12s rRNA and up to 219 bp of the dloop from mtDNA regions for 40 of 57 museum specimens (Table 2). We were able to obtain sequence data for 8 of 22 crocodile mummies. Only the mummified hatchlings from MNHN yielded DNA (Table S3). Our attempts to amplify the larger 12s fragment in the mummy and other museum specimens failed, indicating that there was no contamination with contemporary crocodile DNA. An alignment of the short 12s and d-loop sequences from contemporary specimens found 11 and 14 diagnostic sites, respectively, for the two *C. niloticus* clades (Table 3). Comparison of sequences obtained from the historical specimens to these diagnostic sites enabled us to assign 24 individuals, including all 8 mummy sequences, to the western clade and 16 individuals to the eastern clade (Fig. 1, Table 3). Phylogenetic analysis of the short aDNA dataset recovered a western clade including all mummies and placement of all other museum specimens consistent with the haplotype based clade assignment (Fig. S2, Supporting information).

Haplotype assignments of mummy specimens and well documented collections from the Sudanese Nile valley indicate that the two lineages of *C. niloticus* have had overlapping distributions in the Nile drainage for nearly two millennia (Fig. 1b, Table 3). In addition, derived eastern haplotypes were recovered from two historical specimens from coastal Senegal. Contemporary distributions suggest that little geographical overlap now occurs (Fig. 1a). For example, all contemporary Egyptian specimens possess derived haplotypes, whereas no derived eastern haplotypes have been found in contemporary populations thus far sampled in West Africa.

Discussion

Our total evidence based phylogenetic analysis revealed a cryptic evolutionary lineage within the Nile crocodile. This finding not only clarifies recent and historic disputes regarding both *C. niloticus*' taxonomy and the biogeographic history of the genus, but also stands to improve conservation and management of crocodilian diversity across Africa and elsewhere.

Crocodylus *diversity and taxonomy*

Extant crocodiles are often portrayed as 'living fossils,' reflecting perceptions of morphological homogeneity and evolutionary stasis, but evidence of greater crocodilian diversity and evolutionary dynamism is beginning to emerge. Eaton *et al.* (2009), for example, has found cryptic diversity within the African dwarf crocodiles of the genus *Osteolaemus*. Our results also indicate that greater diversity occurs within the crown genus *Crocodylus* than is currently recognized.

Recognition of subspecies (e.g. Fuchs *et al.* 1974) does not adequately reflect the degree or nature of divergence between the two recovered *C. niloticus* clades. Our findings show that the two *C. niloticus* lineages are distant relatives, and their paraphyletic relationship relative to New World congeners indicates that the two *C. niloticus* clades are not sister taxa. Additionally, fixed differences across sequence-based marker sets and chromosomes, as well as interclade distances, offer a basis for diagnosing the two *C. niloticus* lineages as distinct species (Moritz 1994; Goldstein & DeSalle 2000). Although molecular divergence estimates between members of the genus *Crocodylus* vary by clade and marker, recognized *Crocodylus* species generally exhibit <1% intraspecies divergence and 2.5–7.5% interspecies divergence (White & Densmore 2001; McAliley *et al.* 2006). Similarly, newly diagnosed species within the genus *Osteolaemus* exhibit within-clade divergence of <0.4% and between-clade divergences of 4–16%,

Table 3 Population Aggregation Analysis (PAA) Assigning Archival Specimens to Western or Eastern Clade. Diagnostic nucleotide positions within the short 12s (11 sites) and d-loop (14 sites) sequences. Specimens in bold represent archival material. Eight mummy specimens are highlighted in grey, all correspond to the western lineage. Sequences with question marks across one marker represent failed amplification success for that specimen. D-loop site 206 is an indel event in the eastern clade. The miscoding error observed at d-loop site 226 due to DNA degradation

	Gene region	12s											dloop												
	position	187	193	204	206	209	221	225	229	258	274	303	121	122	128	147	156	201	203	206	209	223	226	227	234	240
Western	Consensus	A	G	A	C	C	A	C	A	T	C	G	A	T	T	C	A	T	A	A	A	T	C	T	C	T
	SAAFedpool	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	BURKINAFAS	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	DRCONGO	.	.	.	.	.	.	.	.	.	.	.	?	?	?	?	?	?	?	?	?	.	.	.	.	.
	GHANA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	GAMBIA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	GAMBIAA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	GAMBIAB	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	IVORYCOAST	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	MAURITANIA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	NIGERIA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	SENEGAL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	RCONGO	.	.	.	.	.	.	.	.	.	.	.	?	?	?	.	.	.	.	.	.	C	.	.	.	.
	KARAMOJAA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	KARAMOJAB	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	MummyHaute	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	C	.	.	.	.
	MummySamA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	C	.	.	.	.
	MummySamB	?	?	?	?	?	?	?	?	?	?	?	.	.	.	.	.	.	.	−	.	C	T	.	.	.
	MummySamC	?	?	?	?	?	?	?	?	?	?	?	.	.	.	.	.	.	.	−	.	C	.	.	.	.
	MummySamD	?	?	?	?	?	?	?	?	?	?	?	.	.	.	.	.	.	.	−	.	C	.	.	.	.
	MummyThebA	?	?	?	?	?	?	?	?	?	?	?	.	.	.	.	.	.	.	−	.	C	T	.	.	.
	mummyThebB	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	C	.	.	.	.
	mummyThebC	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	C	.	.	.	.
	Benin1990	.	.	.	.	.	.	.	.	.	.	.	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	SanghaCAR	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	C	.	.	.	.
	Chad1993	.	.	.	.	.	.	.	.	.	.	.	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	DRCEdz1986	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	C	.	.	.	.
	DRCLukuelu	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	DRCKas1924	.	.	.	.	.	.	.	.	.	.	.	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	CIAssi1885	.	.	.	.	.	.	.	.	.	.	.	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	RCNgou1886	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	C	.	.	.	.
	Matmat1993	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	DRCNE1911	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	C	.	.	.	.
	Oubang1986	?	?	?	?	?	?	?	?	?	?	?	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	Senega1824	?	?	?	?	?	?	?	?	?	?	?	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	Senega1825	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.
	SudMel1922	.	.	.	.	.	.	.	.	.	.	.	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	SudWNA1922	.	.	.	.	.	.	.	.	.	.	.	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	SudWNB1922	.	.	.	.	.	.	.	.	.	.	.	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	Senaga1934	.	.	.	.	.	.	.	.	.	.	.	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Eastern	Consensus	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	G	G	.	C	T	C
	SAAF2	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	G	G	.	C	T	C
	GABONa	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	G	G	.	C	T	C
	NASSERA	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	G	G	.	C	T	C
	NASSERB	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	G	G	.	C	T	C
	NASSERC	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	G	G	.	C	T	C
	NASSERD	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	G	G	.	C	T	C
	MADAGASCNW	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	.	G	.	C	T	C
	MADAGASCSE	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	.	G	.	C	T	C

Table 3 (Continued)

Gene region	12s											dloop													
position	187	193	204	206	209	221	225	229	258	274	303	121	122	128	147	156	201	203	206	209	223	226	227	234	240
MADAGASCAA	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	.	G	.	C	T	C
MADAGASCAB	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	.	A	.	C	T	C
SAFRICA	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	.	A	.	C	T	C
KENYAA	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	G	G	.	C	T	C
KENYAB	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	.	G	.	C	T	C
KENYAC	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	G	G	.	C	T	C
QUEENNP02	?	?	?	?	?	?	?	?	?	?	?	G	C	C	.	G	A	G	C	G	G	.	C	T	C
MURCHISON2	?	?	?	?	?	?	?	?	?	?	?	G	C	C	.	G	A	G	C	G	G	.	C	T	C
LAKEMBURO2	?	?	?	?	?	?	?	?	?	?	?	G	C	C	.	G	A	G	C	G	G	.	C	T	C
ZIMBABWE	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	.	A	.	C	T	C
TANZANIAA	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	.	A	.	C	T	C
TANZANIAB	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	.	A	.	C	T	C
MALAWI	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	.	G	.	C	T	C
Sudan	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Nasser	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Kariba1	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Kariba2	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
DRCNE1912	T	A	G	A	T	T	T	G	A	T	A	G	C	C	C	G	A	G	C	G	G	.	C	T	C
Botswa1967	?	?	?	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
SWCam1966	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
KenGar1960	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
KenNai1919	?	?	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
MadAmA1931	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
MadAmB1931	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
MadAMC1931	?	?	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
madTYP1885	T	A	G	A	T	T	T	G	A	T	A	G	C	C	.	G	A	G	C	.	G	.	C	T	C
vulTYP1822	T	A	G	A	T	T	T	G	A	T	A	G	C	C	T	G	A	G	C	G	G	.	C	T	C
VerTYP1768	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Senega1803	T	A	G	A	T	T	T	G	A	T	A	G	C	C	T	G	A	G	C	G	G	.	C	T	C
SudWNC1922	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
SudWND1922	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
SunWNE1922	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
SudUN1922	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Tanz1972	T	A	G	A	T	T	T	G	A	T	A	?	?	?	?	?	?	?	?	?	?	?	?	?	?

depending on the marker (Eaton *et al.* 2009). In comparison, the two *C. niloticus* clades exhibited 0.3% within-clade and 4% between-clade divergence across 5 kbp (Table S2). Preliminary morphometrics of *C. niloticus* from museum collections representing sites from Kenya and the Congo showing fixed, discrete and non-overlapping continuous character variation (R. Sadlier, unpublished data) also support this conclusion.

That all mummy crocodiles from Thebes and Samoun exhibit the western haplotype suggests both lineages historically occurred in the lower Nile River (Fig. 1). These findings are consistent with early arguments of two *Crocodylus* species in Egypt, including historical accounts that ancient Egyptian priests were cognizant of two forms and selectively used the smaller, more tractable form in temples and ceremonies (Herodotus in Geoffroy Saint-Hilaire 1807). Analysis of museum specimens from more recent collections (Fig. 1b, Table 2) provides additional evidence that both lineages were present in the upper Nile in Sudan until as recently as the 1920s.

Molecular assignment of the eight crocodile mummies to the western *C. niloticus* clade and Geoffroy Saint-Hilaire's (1807) description of a mummified crocodile skull from the same cache as a separate species, *C. suchus*, provides support for ascribing the western *C. niloticus* lineage to this taxon. The description of *C. suchus* included the argument, disputed by Cuvier at the time (Cüvier 1807), that both *C. niloticus* and *C. suchus* were present in the Nile and that the range of *C. suchus* likely extended into the western Sahara (Geoffroy Saint-Hilaire 1807). Geoffroy Saint-Hilaire (1807) went so far as to argue that the distribution of both species likely overlapped in areas of ancient Lake Chad during geologic times.

Crocodylus *biogeography and conservation*

Evidence for cryptic diversity within *C. niloticus* provides key information on the evolution and distribution of the genus *Crocodylus*. Fossils of *Crocodylus checchiai* in Libya (ca. 5–6 mya) (Brochu 2001, 2003) and the Gargano *Crocodylus sp.* (ca. 5–6 mya) of southeastern Italy (Delfino *et al.* 2007) provide evidence of dispersal and diversification within the genus in north Africa and the Mediterranean after the Miocene-Pliocene transition. In light of the fossil record (e.g. Brochu 2003) and estimated divergence dates based on our molecular data, our well-supported phylogenetic hypothesis of a paraphyletic *C. niloticus* bracketing New World congeners provides further support for the hypothesis that the global distribution of *Crocodylus* reflects geologically recent marine and transoceanic dispersal events (Brochu *et al.* 2007; Willis 2009; Meredith *et al.* 2011; Oaks 2011). These findings are consistent with hypothesized transoceanic marine dispersal in other taxa including geckos and parrots (e.g. de Queiroz 2005).

While our divergence estimates are preliminary and partially based on uncertainties in the fossil record for *C. niloticus* in Africa (C. Brochu personal communication), the pattern of divergence we recovered is consistent with many well recognized aspects of African biogeography. The position of Congo Basin samples as basal within the western lineage, and preliminary divergence estimates dating to 8.13 mya for the most recent common ancestor of the western and eastern (including New World species) clades, suggest that the newly identified African *Crocodylus* lineage evolved in the interior of Central Africa during the late Miocene when the closing of the Tethys Sea brought about the climatic trend of increasing aridity we see on the continent today (Axelrod & Raven 1978; Coetzee 1993; Plana 2004). Increasing aridity resulted in the recession of forested areas and the advancement of savannah and woodlands with associated sandy shores necessary for nesting. The contemporary and historical presence of the western lineage at the northeastern margin of the Congo Basin, the Kidepo Valley in northeastern Uganda, and the Sangha River drainage in Central African Republic indicate that dispersal may have begun in a northerly direction and then along an east-west axis facilitated by drainage evolution (Goudie 2005; Drake *et al.* 2011). Further divergence in the western clade occurred throughout the mid to late Pleistocene (0.035–1.43 mya) likely owing to the gradual drying of the 'green Sahara' and subsequent population isolation (Drake *et al.* 2011). During this period, a series of alluvial fans and paleolakes effectively connected the Niger Delta (including the Senegal River) to the Nile basin largely through what was Mega Lake Chad and what is now the Sudd wetland in southern Sudan (Drake *et al.* 2011). Relict populations and rock paintings indicate that crocodile populations were more abundant across northern Africa during wetter climatic periods (de Smet 1999; Shine *et al.* 2001; Drake *et al.* 2011).

Within-lineage genetic structure provides more detailed understanding of connectivity across western Africa. One of the two clades recovered within the western lineage consists largely of Sahelian localities structured by the drying of paleodrainages towards the end of the Pleistocene (Drake *et al.* 2011) (Fig. S1). The other clade is composed of localities in the Upper Guinea Forest Basin countries (e.g. Nigeria, Ghana, Cote d'Ivoire), as well as coastal localities in Senegal and Gambia (Fig. S1). River drainages in this region run north to south draining into the Gulf of Guinea or Atlantic Ocean, and therefore have had little connection with paleodrainages of the Sahara. The observed phylogenetic structure also likely reflects drainage isolation with infrequent marine dispersal, a pattern seen in some coastal fishes (e.g. Falk *et al.* 2003; Agnese *et al.* 2006). Nile crocodiles are abundant in coastal lagoons in this region and are regularly observed in marine environments (Shirley *et al.* 2009; Fergusson 2010).

Similarly, the eastern clade of *C. niloticus* is broken into two sister groups dating to around 3.274 mya with likely origins in the Nile valley. Prior analyses of eastern populations based on nuclear markers revealed substantial sub-structuring corresponding to major barriers to dispersal such as the Mozambique Channel (East Africa and Madagascar), and to river drainages in Kenya, Tanzania, Zimbabwe and South Africa (the Turkana, Ruaha, Zambezi and Limpopo river basins, respectively) (Hekkala *et al.* 2009). It is possible that the geographic structure exhibited by eastern *C. niloticus* may be related to patterns of natal philopatry-associated breeding and nesting behaviors (Hekkala *et al.* 2009). Similar patterns of sub-structuring by drainage basin have been observed in faunal assemblages found in East African forest remnants (Azeria *et al.* 2007).

Our recovery of the eastern haplotype in two samples from western Central Africa (i.e. the Ogooué Basin—Gabon and Cameroon) likely reflects northward dispersal from coastal Angola and the Kunene River. The Cameroon Volcanic Line is a major biogeographic feature separating this region from coastal West Africa (Cantagrel *et al.* 1978; Lee *et al.* 1994; Meyers *et al.* 1998), and a similar pattern occurs in the *Osteolaemus* dwarf crocodiles (Eaton *et al.* 2009).

On a continental scale, the cryptic east/west split found in our study of African *Crocodylus* parallels patterns of differentiation observed between sister taxa in several African faunal assemblages following the formation of the Rift Valley (de Menocal 2004; Moodley & Bruford 2007). However, the geographic distributions of

the ancestral and derived lineages (Fig. 1a, b) belie a history of greater sympatry in Africa. The occurrence of the derived lineage in historical specimens from Senegal suggests the possibility of either greater sympatry in western Africa in the past or a pattern of coastal dispersal by the Eastern lineage, though no contemporary specimens from West Africa to date, coastal or otherwise, support either argument (Fig. 1). Individuals from historical collections from the Sudanese Nile valley (1822–1922) and northeastern DRC (1911–1912) also possess both lineages. While further sampling in Sudan and NE DRC is needed to determine the extent of sympatry today, the presence of the western clade in the Kidepo Valley (Uganda) and anecdotal evidence of similar crocodile populations in Ethiopia suggests that the western clade is still distributed in this region though it may be restricted to marginal habitats.

Previously, researchers using molecular data from paleontological collections have shown evidence that genetic diversity in wide ranging species has been lost over historical and paleoecological time periods (Ramakrishnan & Hadley 2009 and references therein). This growing field has been termed 'phylochronology' due to the emphasis on reconstructing patterns of genetic variation over time. Much of this work has focused on Holocene patterns of faunal turnover and range contractions in northern latitudes (Ramakrishnan & Hadley 2009; examples therein, e.g. Shapiro *et al.* 2004; Hofreiter *et al.* 2004). While these studies are invaluable in advancing understanding of the genetic consequences of environmental change, our study reveals a much more recent pattern of local extirpation with potentially global consequences for loss of crocodilian biodiversity.

Conclusion

This study emphasizes once again the utility of non-traditional archival specimens in contributing to our understanding of evolutionary relationships and biogeographic history (Leonard 2008). As techniques for accessing nucleic acids from archival materials become more readily and reliably available, materials found in ever more diverse repositories stand to provide greater insight into changes over time related to natural and anthropogenic processes. Our success in accessing DNA from archival materials adds to the growing body of work demonstrating the role of museum collections as banks of 'ancient' DNA that can be used to establish baseline genetic profiles against which change can be measured (Leonard 2008; Ramakrishnan & Hadley 2009 and references therein). However, use of archival materials is not without risk (Cooper & Poinar 2000). Many researchers examining genetic characteristics of paleomaterial have difficulty retrieving and authenticating ancient DNA. The mummified crocodile hatchings, with the exception of the 'pre-dynastic' hatchling from PHM, proved to be an exceptional source for ancient DNA. The specimens came from dry, sealed, relatively cool burial chambers and are young (only 1 800–2 200 years old) in comparison to source materials used in many other ancient DNA studies (e.g. Hofreiter *et al.* 2004; Shapiro *et al.* 2004). Importantly, our samples have two additional, uniquely crocodilian advantages over samples comprised of mammalian bone and mummy tissue: nucleated red blood cells and a thick keratinized skin layer. Both of these attributes likely serve as sources and protective repositories for mtDNA.

Our combined analyses of museum and contemporary specimens indicate that, as formulated, major national and international conservation agreements intended to promote sustainable harvest of Nile crocodiles may instead accelerate extirpation because quotas and translocation policies are based on erroneous taxonomy and assumptions of genetic homogeneity. This is particularly relevant in countries that harbour populations of both lineages and have long running harvest programs (e.g. Uganda) or are looking to initiate new harvest programs (e.g. Ethiopia and Sudan). The newly discovered evolutionary lineage of African *Crocodylus* is particularly vulnerable to extinction because of its relative rarity and restricted occurrence in countries where illegal harvest of skins, the bushmeat trade, and damage to wetlands are largely unregulated (Shirley *et al.* 2009). Taking precautionary measures, such as recognizing the ancestral lineage as *C. suchus* on the IUCN Red List and reviewing its status, could reduce further loss of at-risk populations.

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Author contributions

EH and MHS, who contributed equally to this work and are considered co-primary authors, designed the study, and conducted all lab work and phylogenetic analyses. EH collected samples from Madagascar and conducted all museum sampling and aDNA work. MHS collected all samples from Ghana, Cote-d'Ivoire, Senegal, Gambia, Uganda and Egypt. GA and RD, and JDA are the dissertation supervisors of EH and MHS, respectively, and contributed to the development of methods and provided funding support. JT was an avid conservationist and the MSc advisor for MHS. He contributed significantly to our understanding of the taxonomic history of Nile crocodiles, sampling strategy, design of fieldwork, and funding support. SC and MH conducted all karyotype analyses. KV contributed to the karyotype analysis of captive animals. MB contributed analytical expertise and lab support. Sampling protocols were reviewed by the University of Florida IACUC (#E-423).

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M.H.S.'s research utilizes multiple inferential tools to elucidate population-level processes over different temporal and spatial scales to facilitate the conservation of wildlife in Africa and elsewhere. He is particularly interested in the interaction between historic, landscape features and contemporary human pressures in structuring wildlife populations.

Data accessibility

DNA sequences: DRYAD entry (datadryad.org; doi:10.5061/dryad.s1m9h)

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Gene regions and protocols used for amplification of mtDNA and nuclear introns for *Crocodylus niloticus* and affiliated specimens used in the study

Table S2 Estimated Molecular Divergence. Mean distance estimates with S.E. for the full, concatenated dataset (below diagonal) and mtDNA-only dataset (above diagonal). Values in the diagonal are intragroup mean distance estimates with S.E. for the full, concatenated dataset (left) and mtDNA-only dataset (right)

Table S3 All mummy specimens examined for this study. Locality and date information is from museum accession notes unless otherwise noted

Fig. S1 Estimated divergence dates for the two *Crocodylus niloticus* clades under a relaxed clock model as implemented in BEAST v.4.3. The displayed estimates for mean divergence date and 95% confidence intervals are based on the full dataset partitioned by coding region with subsequent codon position partitioning.

Fig. S2 Phylogenetic tree resulting from maximum likelihood analysis of concatenated 12s and d-loop short fragments for contemporary and archival specimens. Mummy specimens have blue terminal labels.

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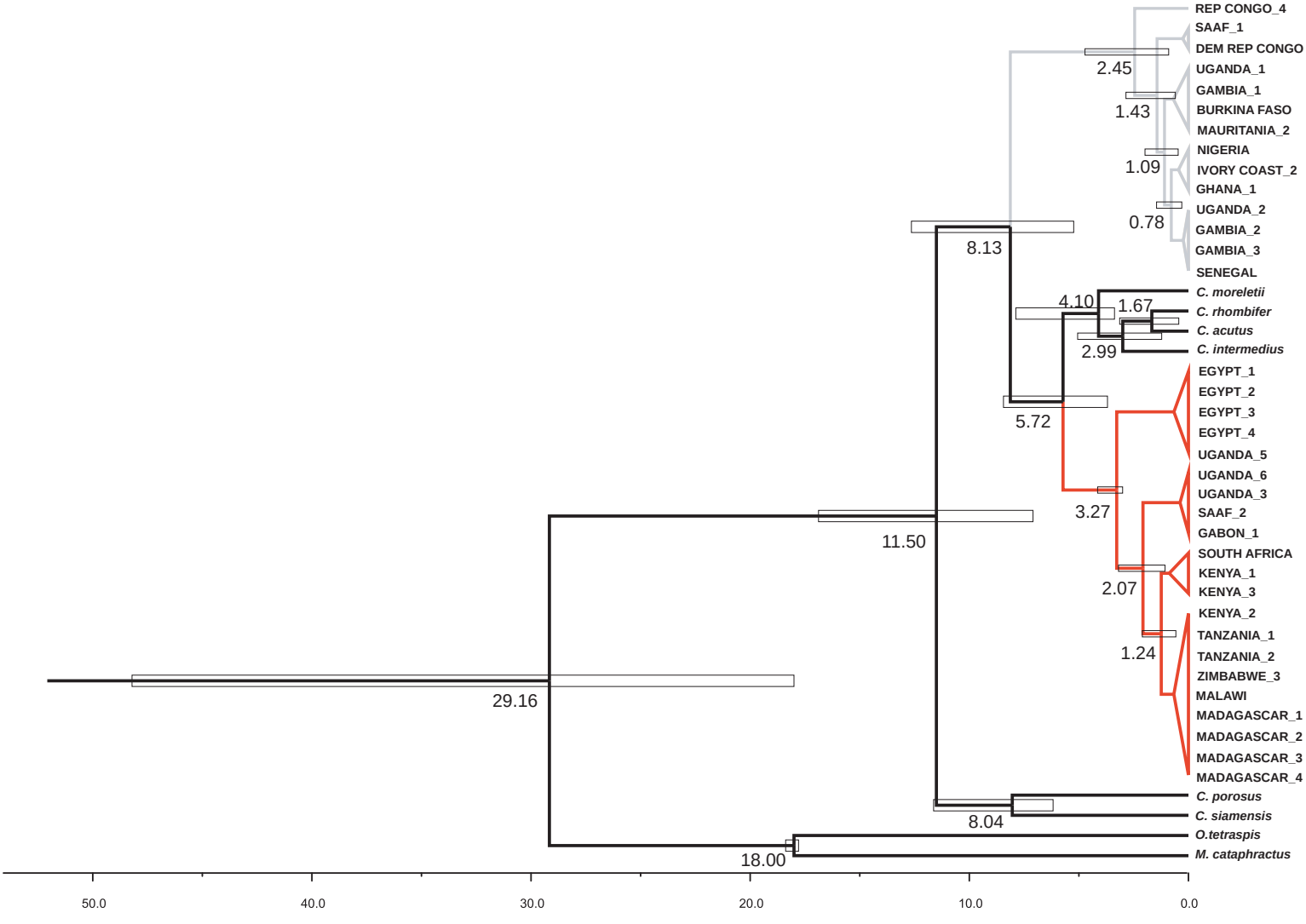
REP CONGO_4
SAAF_1
DEM REP CONGO
UGANDA_1
GAMBIA_1
BURKINA FASO
MAURITANIA_2
NIGERIA
IVORY COAST_2
GHANA_1
UGANDA_2
GAMBIA_2
GAMBIA_3
SENEGAL
C. moreletii
C. rhombifer
C. acutus
C. intermedius
EGYPT_1
EGYPT_2
EGYPT_3
EGYPT_4
UGANDA_5
UGANDA_6
UGANDA_3
SAAF_2
GABON_1
SOUTH AFRICA
KENYA_1
KENYA_3
KENYA_2
TANZANIA_1
TANZANIA_2
ZIMBABWE_3
MALAWI
MADAGASCAR_1
MADAGASCAR_2
MADAGASCAR_3
MADAGASCAR_4
C. porosus
C. siamensis
O.tetraspis
M. cataphractus
2.45
1.43
1.09
0.78
8.13
4.10
1.67
2.99
5.72
3.27
11.50
2.07
1.24
29.16
8.04
18.00
50.0
40.0
30.0
20.0
10.0
0.0

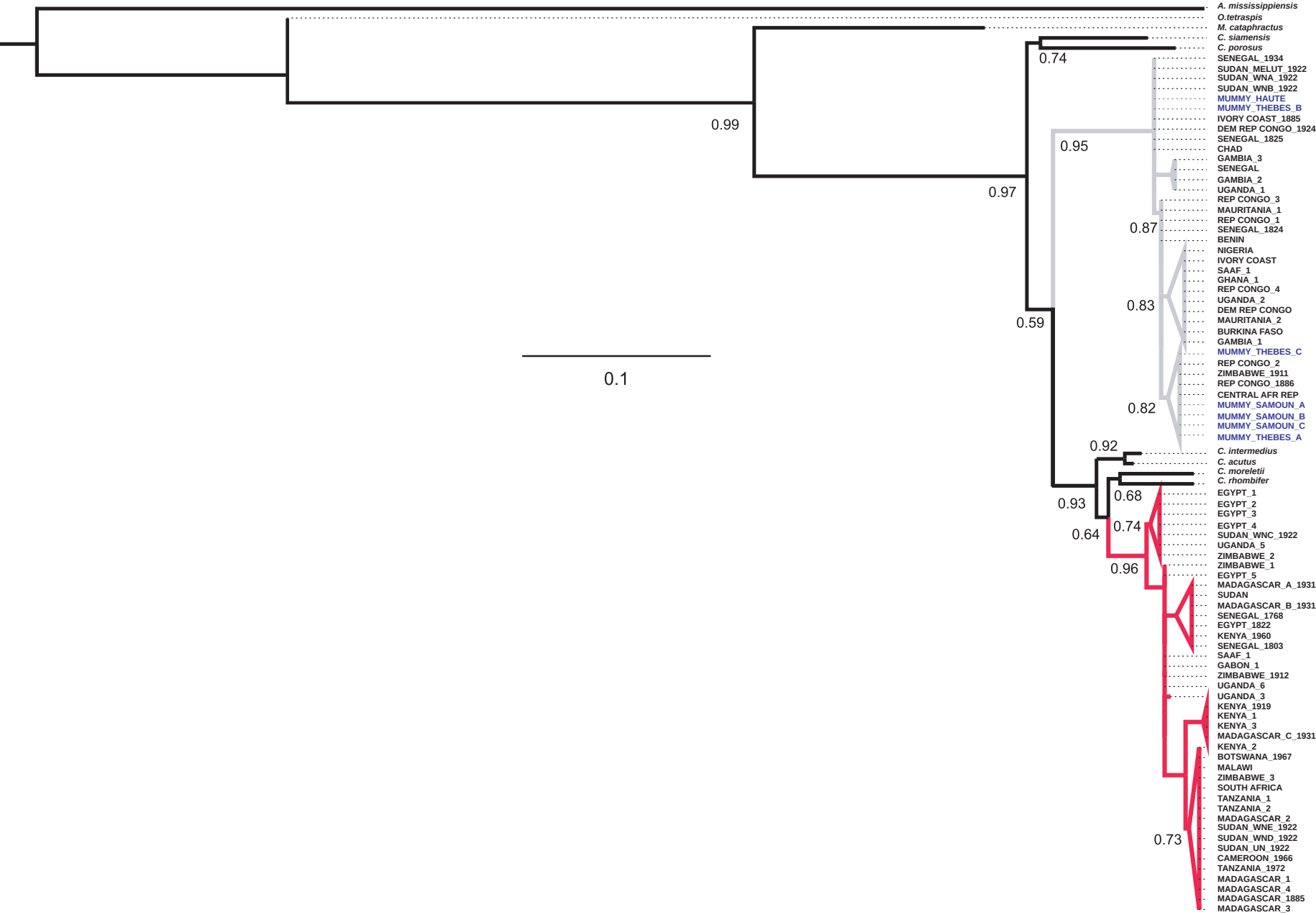
A. mississippiensis
O.tetraspis
M. cataphractus
C. siamensis
C. porosus
SENEGAL_1934
SUDAN_MELUT_1922
SUDAN_WNA_1922
SUDAN_WNB_1922
MUMMY_HAUTE
MUMMY_THEBES_B
IVORY COAST_1885
DEM REP CONGO_1924
SENEGAL_1825
CHAD
GAMBIA_3
SENEGAL
GAMBIA_2
UGANDA_1
REP CONGO_3
MAURITANIA_1
REP CONGO_1
SENEGAL_1824
BENIN
NIGERIA
IVORY COAST
SAAF_1
GHANA_1
REP CONGO_4
UGANDA_2
DEM REP CONGO
MAURITANIA_2
BURKINA FASO
GAMBIA_1
MUMMY_THEBES_C
REP CONGO_2
ZIMBABWE_1911
REP CONGO_1886
CENTRAL AFR REP
MUMMY_SAMOUN_A
MUMMY_SAMOUN_B
MUMMY_SAMOUN_C
MUMMY_THEBES_A
C. intermedius
C. acutus
C. moreletii
C. rhombifer
EGYPT_1
EGYPT_2
EGYPT_3
EGYPT_4
SUDAN_WNC_1922
UGANDA_5
ZIMBABWE_2
ZIMBABWE_1
EGYPT_5
MADAGASCAR_A_1931
SUDAN
MADAGASCAR_B_1931
SENEGAL_1768
EGYPT_1822
KENYA_1960
SENEGAL_1803
SAAF_1
GABON_1
ZIMBABWE_1912
UGANDA_6
UGANDA_3
KENYA_1919
KENYA_1
KENYA_3
MADAGASCAR_C_1931
KENYA_2
BOTSWANA_1967
MALAWI
ZIMBABWE_3
SOUTH AFRICA
TANZANIA_1
TANZANIA_2
MADAGASCAR_2
SUDAN_WNE_1922
SUDAN_WND_1922
SUDAN_UN_1922
CAMEROON_1966
TANZANIA_1972
MADAGASCAR_1
MADAGASCAR_4
MADAGASCAR_1885
MADAGASCAR_3
0.99
0.74
0.95
0.97
0.87
0.83
0.59
0.82
0.92
0.68
0.93
0.74
0.64
0.96
0.73
0.1

Table S1

Gene	PCR Reaction Cocktail						PCR Cycle Conditions										
	Vol.	Reaction Buffer	$MgCl_2$	dNTP's	Primer	Taq	Extended Denature		Denature		Anneal		Extension		Extended Extension		#
	µL	*5X*	*mM*	*mM*	*µM*	*U/µM*	*Minutes*	*˚C*	*Minutes*	*˚C*	*Minutes*	*˚C*	*Minutes*	*˚C*	*Minutes*	*˚C*	*#*
12S	15	1	1.5	0.2	0.5	0.03	4:00	94	1:00	94	1:00	52	1:30	74	4:00	72	35
12s (short)	25	Illustra puretaq beads					5:00	94	1:00	94	1:00	52	1:30	72	4:00	72	35
16S	25	Illustra puretaq beads					5:00	94	1:00	94	1:00	52	1:30	72	4:00	72	33
Control Region/dloop	15	1	1.5	0.2	0.5	0.03	4:00	94	1:00	94	1:00	54	1:30	72	4:00	72	35
d-dloop short	25	Illustra puretaq beads			1		4:00	94	1:00	94	1:00	54	1:30	72	4:00	72	35
ND4	25	Illustra puretaq beads					5:00	94	1:00	94	1:00	52	1:30	72	4:00	72	33
Wancy	15	1	1.75	0.2	0.5	0.03	4:00	94	1:00	94	1:00	55	1:30	72	4:00	72	35
Rag-1	15	1	1.5	0.2	0.5	0.03	4:00	94	1:00	94	1:00	56	1:30	72	4:00	72	35
Trop	20	0.85	1.5	0.2	0.5	0.03	4:00	94	1:00	94	1:15	56	1:30	76	4:00	74	35
OD	20	0.9	1.5	0.2	0.5	0.03	4:00	94	1:00	94	1:00	54	1:30	74	4:00	72	35
S6	15	0.9	1.5	0.2	0.5	0.03	4:00	94	1:00	94	1:00	60	1:30	74	4:00	72	35

Table S2	Eastern *C. niloticus*		Western *C. niloticus*		New World		Asia		*M. cataphractus*	*O. tetraspis*	*A. mississippiensis*
Eastern *C. niloticus*	0.003 ±0.0008	0.007 ±0.00169	0.045 ±0.00921		0.032 ±0.00647		0.066 ±0.01247		0.125 ±0.02228	0.166 ±0.03081	0.484 ±0.14319
Western *C. niloticus*	0.044 ±0.01136		0.004 ±0.00094	0.007 0.002	0.057 ±0.01158		0.065 ±0.01239		0.123 ±0.02240	0.174 ±0.03332	0.506 ±0.16007
New World	0.039 ±0.00892		0.056 ±0.01325		0.023 ±0.00463	0.031 ±0.00577	0.071 ±0.01329		0.135 ±0.02426	0.178 ±0.03282	0.514 ±15967
Asia	0.075 ±0.01670		0.067 ±0.01603		0.076 ±0.01680		0.076 ±0.0149	0.077 ±0.01374	0.138 ±0.02463	0.188 ±0.03480	0.492 ±0.15171
Mecistops cataphractus	0.144 ±0.03427		0.144 ±0.03492		0.140 ±0.03296		0.153 ±0.03589		N/A	0.155 ±0.02876	0.513 ±0.15830
Osteolaemus tetraspis	0.175 ±0.04625		0.162 0.042		0.158 ±0.04152		0.197 ±0.05273		0.159 ±0.03966	N/A	0.471 ±0.13390
Alligator mississippiensis	0.293 0.086		0.332 ±0.11862		0.332 ±0.10341		0.396 ±0.14893		0.338 ±0.10481	0.316 ±0.09330	N/A

Museum	Specimen Number	Terminal Label	Specimen Name	Site Number	Locality	Country	Collector	Date Collected	Haplotype
MNHN	1986_1475	MUMMY_SAMOUN_A	mummySamA	8	Mummy - Grottes de Samoun	Egypt	Gervais	200BC-200AD*	W
MNHN	1986_1478	MUMMY_SAMOUN_B	mummySamB	8	Mummy - Grottes de Samoun	Egypt	Gervais	200BC-200AD*	W
MNHN	1986_1480	MUMMY_SAMOUN_C	mummySamC	8	Mummy - Grottes de Samoun	Egypt	Pariset	200BC-200AD*	W
MNHN	1986_1471	MUMMY_THEBES_A	mummyThebA	7	Mummy - Grottes de Thebes	Egypt	Cailloud - collected 1820s	200BC-200AD*	W
MNHN	1986_1473	MUMMY_THEBES_B	mummyThebB	7	Mummy - Grottes de Thebes	Egypt	Cailloud - collected 1820s	200BC-200AD*	W
MNHN	1986_1479	MUMMY_THEBES_C	mummyThebC	7	Mummy - Grottes de Thebes	Egypt	Cailloud - collected 1820s	200BC-200AD*	W
MNHN	1886_445	MUMMY_HAUTE	MummyHaute	7	Mummy, Haute Egypt	Egypt	V. Schoelcher	200BC-200AD*	W
PHM	620101	N/A	PHM620101	N/A	Mummy unknown	Egypt	Unknown	pre-dynastic	ND
PHM	55121	N/A	PHM55121	N/A	Mummy unknown	Egypt	Unknown	pre-dynastic	ND
PHM	514	N/A	PHM514	N/A	Mummy unknown	Egypt	Unknown	pre-dynastic	ND
BM	35734	N/A	BM35734	N/A	Mummy Manfalut	Egypt	E.J. Andrews	Roman	ND
BM	35726	N/A	BM35726	N/A	Mummy unknown	Egypt	Unknown	pre-dynastic	ND
BM	35747	N/A	BM35747	N/A	Mummy Manfalut	Egypt	Unknown	pre-dynastic	ND
BM	35751	N/A	BM35751	N/A	Mummy Manfalut	Egypt	Unknown	pre-dynastic	ND
BM	6837	N/A	BM6837	N/A	Mummy Manfalut	Egypt	E.J. Andrews	Roman	ND
BM	6847	N/A	BM6847	N/A	Mummy Manfalut	Egypt	E.J. Andrews	Roman	ND
Upenn	E521	N/A	UpennE521	N/A	Mummy unknown	Egypt	Unknown	ND	ND
Upenn	2965563	N/A	Upenn2965563	N/A	Mummy-Dindereh	Egypt	Cox Expedition 1918	ND	ND
Upenn	E2832	N/A	UpennE2832	N/A	Mummy-Tel El Yehudiyeh	Egypt	Flinders Petrie	ND	ND
Upenn	L12112	N/A	UpennL12112	N/A	Mummy-Maabdah (Samoun)	Egypt	Unknown	Late period	ND
Upenn	L12113	N/A	UpennL12113	N/A	Mummy-Thebes?	Egypt	G.R. Glidden 1848	Late period	ND

MNHN= Musee National d'Histoire Naturelle, PHM=Phoebe Heart Museum UCBerkeley, BM=British Museum, Upenn=Penn Museum

* estimated dates as per S. Ikram Cairo Museum