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The secrets of Sobek – A crocodile mummy mitogenome from ancient Egypt

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ABSTRACT

Previous investigations of genetic diversity across the distribution of the Nile crocodile (*Crocodylus niloticus*) confirmed the existence of two genetically distinct species of true crocodile (genus *Crocodylus*) in Africa. These taxa correspond roughly to an eastern/southern African species (*Crocodylus niloticus*) and a central/western African species (*Crocodylus suchus*). Analysis of historical museum specimens demonstrated that both species existed concurrently in the Sudanese Nile until the early 20th century and genetic analyses of historical museum specimens of mummified crocodile hatchlings from Egyptian tombs located along Egyptian Nile were found to be *C. suchus*.

Here we present the first assessment of mitogenomic data from an adult Egyptian crocodile mummy from a center of crocodile worship and identify this specimen as *C. suchus*. Our data suggest that *C. suchus* was selectively chosen for mummification and support an accurate Egyptian cultural taxonomy as described by Herodotus in the fourth century BC and used by Etienne Geoffroy Saint-Hilaire to describe *Crocodylus suchus* in 1807. *Crocodylus suchus* has experienced a range contraction possibly due to climate change and the drying of the Sahara over the recent past. Our data identifying an adult crocodile mummy as *C. suchus* might indicate the historical natural presence of this species in the Egyptian Nile along with *C. niloticus*. Additional samples of crocodiles from both bioarchaeological and paleontological contexts will be required to confirm this.

1. Introduction

The French naturalist Etienne Geoffroy Saint-Hilaire made an effort to collect and document evidence for variation between Egyptian animal mummies and their modern relatives with the intent to prove that species changed in response to shifting environmental conditions (Le Guyader, 2004; Curtis et al., 2018). During the Napoleonic Expedition to Egypt (1798–1801), he assembled a diverse collection of animal mummies including cats (Richardin et al., 2017), ibises (Wasef et al., 2015), shrews (Woodman et al., 2017), and crocodiles (Geoffroy Saint-Hilaire, 1807). Geoffroy Saint-Hilaire was particularly interested in comparing anatomical features and morphological variation among ancient and modern representatives of these species to show that features were mutable across time. Over the last two decades researchers analyzing the remains of animals from archaeological sites have made great progress in recovering genomic data to better understand how the distribution of variation in wild and domestic species is related to human use and manipulation of natural resources (Vilstrup et al., 2013). Two hundred years after Geoffroy Saint-Hilaire's hypotheses, modern sequencing technologies have allowed researchers to demonstrate that ancient DNA is present in the remains of animal mummies and can be directly compared with data from modern taxa (Curtis et al.,

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2018; Hekkala et al., 2011; Kurushima et al., 2012).

In one such example, researchers used Sanger sequencing of nuclear and mitochondrial gene regions to show that the familiar Nile crocodile found throughout Africa actually consists of two species. One, the familiar Nile crocodile (*Crocodylus niloticus*) is distributed throughout East and southern Africa, and a second, cryptic species that is now found in West and Central Africa (Hekkala et al., 2011). In that study, short mitochondrial DNA sequences recovered from a set of mummified hatchling crocodiles from Thebes and Grotte De Samoun (now referred to as Ma'abdeh) in Egypt were found to match those from extant crocodile populations in West and Central Africa. The authors identified a previously proposed species name, *Crocodylus suchus,* from the literature (Geoffroy Saint-Hilaire, 1807). This species' original description was based on a juvenile crocodile mummy collected from Thebes during Napoleon's expedition to Egypt and illustrated in plate 55 of the folio version of the "Description de L'Egypt" (Geoffroy Saint-Hilaire, 1807; Jomard and Jacotin, 1818; Hekkala et al., 2011).

Today the resurrected species, *Crocodylus suchus,* exists in populations throughout western Africa, including the Congo Basin, extreme northwestern Uganda (Shirley et al., 2015, Cunningham et al., 2016), the Awash River basin of Ethiopia (Siege and Koch 2017), and in an isolated guelta in the Ennedi plateau of Chad (Schmitz et al., 2003). Genetic barcoding evidence from additional historical museum collections, however, confirmed that this species' distribution extended to Melut in the White Nile until 1922 (Fig. 2, and Fig. 1a and b in Hekkala et al., 2011). Extensive surveys over the past decade have failed to encounter this species among extant crocodile populations in Egypt (Shirley et al., 2012, 2015).

Geoffroy Saint-Hilaire hypothesized in his 1807 paper that the species would likely have been found in both the Nile River Valley and westward throughout the Sahara during a period of time when northern Africa was wetter (Geoffroy Saint-Hilaire, 1807). Following Geoffroy Saint-Hilaire and based on the confirmed presence of the hatchling *C. suchus* mummies from Thebes and Ma'abdeh grotto in Egypt, we propose that 1) Ancient Egyptian folk taxonomy accurately assessed the existence of two differentiated forms of African *Crocodylus*, and 2) the distribution of the sacred species was more extensive in the Sahara and the Nile basin during past millennia.

In order to further evaluate the taxonomic identity of crocodile mummies and also help establish the historical distributions of *C. suchus* and *C. niloticus* in ancient Egypt, we use next-generation sequencing of enriched DNA libraries to collect mitogenomic data from an adult crocodile mummy (c. 3rd cen. BC-AD 2nd cen.) indicated as being from the temple of Kom Ombo (Egypt), a site of worship for the crocodile headed god Sobek (Ikram, 2015).

Fig. 1. The crocodile mummy (head only) on display in-situ in the Natural History Museum-G, Salzkammergut-Austria (inset full length).

2. Materials

2.1. Crocodile mummy samples

We sampled a crocodile mummy currently housed at the Natural History Museum of the Salzkammergut, Austria (NMSG-A) for bone and muscle tissue for sequencing. Based both on the mummy's size and archival documents provided by the museum curator, this specimen likely originated from the site of Kom Ombo in Egypt, which hosts a Ptolemaic temple and a nearby animal cemetery at al-Shutb, noted for its large-size mummified crocodiles. The temple is dedicated to the crocodile headed god, Sobek, a major Egyptian deity who was believed to confer fertility and strength. The specimen, a gift of the Egyptian government to Professor Otto Stober in 1960–61, was displayed in the Moor Museum in Bad Neyhardting until the museum's dissolution in 2000. It was then transferred to NMSG-A, where it is currently on display (Fig. 1). There is little else known regarding the specimen's provenance, however, features of the mummification type and wrapping match those of other in-situ Kom Ombo crocodile mummies, an area that is the major source for museum specimens of this size (for example, British Museum EA 38562; Egyptian Museum Cairo CG 29,628 and CG 29630).

As was typical for crocodile mummies, the mummy was desiccated, anointed with oils and resinous materials, and wrapped in linen bandages permeated by resinous materials (Ikram and Iskander, 2002). Based on the specimen's total length (2.5 m) and evidence of an everted mummified penis, the crocodile mummy likely represents a reproductively mature male.

2.2. Vouchered contemporary and crocodilian museum samples.

We included vouchered samples from live animals (contemporary) and from natural history collections (archival) to provide a comparative mitogenomic data set for phylogenetic placement of the ancient (aDNA) mummy sequences (See Table 1 for specimen details). To facilitate reconstruction of the phylogenetic relationships of the mummy mitogenome we included all members of the genus *Crocodylus* and added *Osteolaemus tetraspis* data from Genbank as an outgroup. Museum specimens are hereafter referred to as "archival" samples to differentiate between these and the "ancient" mummy samples.

3. Laboratory methods

3.1. Genomic DNA extraction and library preparation

3.1.1. Mummy samples

Samples of the crocodile mummy were collected on site at the NMSG-A in Bad Ebensee, Austria during exhibition renovations. Using sterilized large bore drill bits and foil reservoirs, one dried tissue sample (sample KomOmbo21, replicate 1) and one bone sample (sample KomOmbo22, replicate 2) were each collected from the scapular region, which was initially wiped with dilute bleach (0.5%) and air-dried. To reduce potential DNA damage from heating or vibration the drill was set on the slowest setting. The first sample was taken from dried muscle and the drill bit was replaced with a new sterile bit and a sample of exposed bone was removed from the same site after an additional surface sterilization. For each replicate an approximately 30–40 mg sample of tissue (KomOmbo 21) or of bone (KomOmbo 22) was collected in a sterile foil reservoir. For mock sampling controls for each replicate a blank sterile 1.5 ml Eppendorf tube was held adjacent to the work area and touched with sterile collection materials for each sample collected. After the destructive sampling the specimen was repaired for display. The samples (KomOmbo21 and 22 and blank 21 and 22 respectively) were then shipped to, and processed in, a clean lab facility at the University of Copenhagen.

Prior to extraction the surfaces of tissue and bone samples were

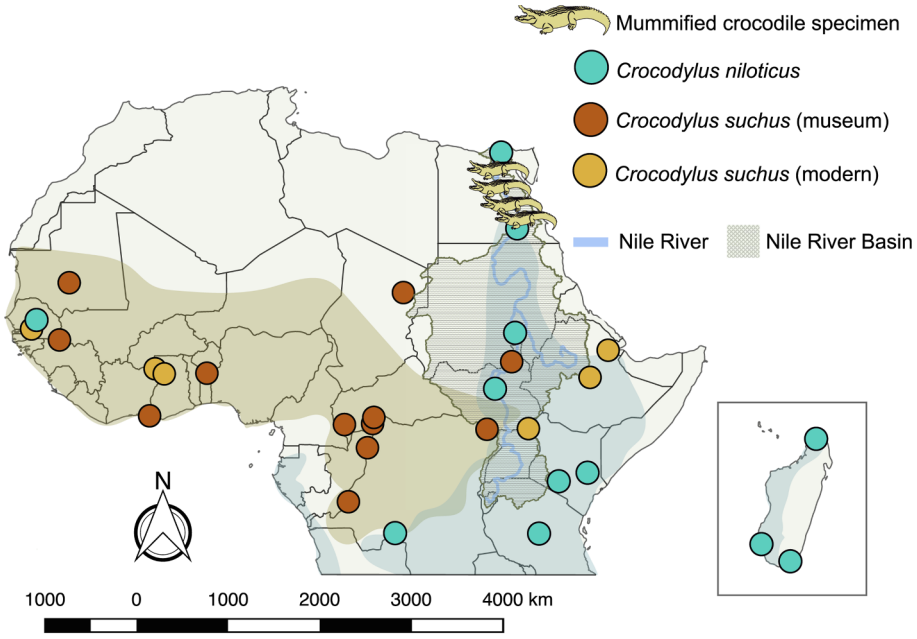

Fig. 2. Map of the Nile River drainage basin and historical and current distributions of *Crocodylus niloticus* and *C. suchus* and major crocodile mummy sites discussed in the text. Current distributions of *C. niloticus* (light blue) and *C. suchus* (tan) modified from Shirley et al. (2015). Archival specimens of *C. niloticus* (blue) and *C. suchus* (brown) are from Hekkala et al. (2011). Extant *C. suchus* sample locations (Shirley et al., 2015; Cunningham et al., 2016) are indicated in gold. Madagascar is shown in the inset.

Table 1
Contemporary and archival crocodilian specimens sequenced for comparison to the Salzkammergut mummy mitogenome. For contemporary samples acronym (AMCC) is the Ambrose Monel Cryo Collection at the American Museum of Natural History, (EH) is E. Hekkala, (MHS) M.H. Shirley, (AZA) is Association of Zoos and Aquaria and (SAAF) is St. Augustine Alligator Farm Zoological Park, St. Augustine Florida. For Archival samples from museum collections, acronym (AMNH) is the American Museum of Natural History, Department of Herpetology and (NMSG-A) is the Natural History Museum of the Salzkammergut, Austria.

Organism	Sample name	DNA state	Source	Accession	Locality
Crocodylus acutus	AMNH7120	museum archival	AMNH	MT727010	No Data
Crocodylus acutus	NC_015647.1	modern	Genbank	NC_015647.1	NA
Crocodylus intermedius	L193	modern	SAAF	MT727027	Venezuela
Crocodylus johnsoni	L122	modern	SAAF	MT727018	Captive Bred
Crocodylus johnsoni	L072	modern	SAAF	MT727019	Captive Bred
Crocodylus johnsoni	NC_015238.2	modern	Genbank	NC_015238.2	NA
Crocodylus mindorensis	L119	modern	SAAF	MT727007	Phillipines (Captive Bred)
Crocodylus mindorensis	L080	modern	SAAF	MT727017	Phillipines (Captive Bred)
Crocodylus moreletii	NC_015235.1	modern	Genbank	NC_015235.1	NA
Crocodylus niloticus	amnh142496	museum archival	AMNH	MT727003	Madagascar
Crocodylus niloticus	amnh7130	museum archival	AMNH	MT727004	No Data
Crocodylus niloticus	amnh73047	museum archival	AMNH	MT727005	Kenya
Crocodylus niloticus	AMNH71192	museum archival	AMNH	MT727006	Madagascar
Crocodylus niloticus	AMNH29291	museum archival	AMNH	MT727009	No Data
Crocodylus niloticus	mad352	modern	EH	MT727013	Madagascar (LacBemaba)
Crocodylus niloticus	Ank1	modern	EH	MT727014	Madagascar (Ankarana)
Crocodylus niloticus	Ank14	modern	EH	MT727016	Madagascar (Ankarana)
Crocodylus niloticus	Tana3	modern	EH	MT727021	Kenya (Tana River)
Crocodylus niloticus	NEDE02	modern	MHS	MT727022	Egypt (Lake Nasser)
Crocodylus niloticus	NEDE03	modern	MHS	MT727028	Egypt (Lake Nasser)
Crocodylus novaeguineae	L184	modern	SAAF	MT727020	Papua New Guinea (Fly River-Captive Bred)
Crocodylus novaeguineae	L088	modern	SAAF	MT727023	Papua New Guinea (Fly River-Captive Bred)
Crocodylus palustris	AMCC110220	modern	AMCC	MT727012	No Data
Crocodylus palustris	NC_014706.1	modern	Genbank	NC_014706.1	NA
Crocodylus porosus	NC_008143.1	modern	Genbank	NC_008143.1	NA
Crocodylus rhombifer	L138	modern	SAAF	MT727024	Captive Bred
Crocodylus rhombifer	L139	modern	SAAF	MT727025	Captive Bred
Crocodylus rhombifer	L140	modern	SAAF	MT727026	Captive Bred
Crocodylus siamensis	LZ013	modern	AZA	MT727015	Captive Bred
Crocodylus suchus	AMNH127255	museum archival	AMNH	MT727008	No Data
Crocodylus suchus	AMNH118718	museum archival	AMNH	MT727011	No Data
Crocodylus suchus	NKRP03	modern	MHS	MT727029	Gambia
Crocodylus suchus	NKRP04	modern	MHS	MT727030	Gambia
Crocodylus suchus	NKabak04	modern	MHS	MT727031	Guinea
Crocodylus suchus	KomOmbo_Mummy21	Ancient	NMSG-A	MT727032	Egypt (Likely Kom Ombo)
Crocodylus suchus	KomOmbo_Mummy22	Ancient	NMSG-A	MT727033	Egypt (Likely Kom Ombo)
Osteolaemus tetraspis tetraspis	NC_009728.1	modern	Genbank	NC_009728.1	Cameroon

rinsed for five seconds in a 5% bleach solution and then rinsed three times in ultrapure water and dried. Each sample (whether bone or tissue) was processed along with blank controls at each step for a total of four samples. The ancient DNA extraction protocol for bone (KomOmbo22) broadly followed Dabney (Dabney et al., 2013) with minor modifications. Approximately 300 mg of powdered bone was digested overnight at 37 °C in 1 ml extraction buffer (0.45M EDTA, 0.25 mg ml proteinase K, pH 8.0) while rocking. The tissue sample (KomOmbo21) was digested without an initial EDTA soaking step. Approximately 300 mg of dried tissue was minced and digested overnight at 37 °C in 1 ml extraction buffer (0.45M EDTA, 0.25 mg ml proteinase K, pH 8.0) while rocking. Residue for each sample was then pelleted by centrifugation at maximum speed (16,000g) and the supernatant was transferred to 15 ml volume binding buffer in Zymo large format spin column reservoirs attached to Qiagen MinElute spin columns. After resting for 15 min, the modified large format columns were nested in 50 ml falcon tubes and centrifuged at low speed (600g) for 20–30 min. DNA bound to MinElute columns was washed twice with 500 μL PE buffer and eluted by adding 20 μL elution buffer in two rounds of centrifugation for a total volume of 40 μL. Blanks for each replicate followed the extraction protocol for the relevant sample.

Recovery of DNA was assessed using Qubit dsDNA HS Assay kit and double stranded DNA libraries were produced from the DNA extracts using the blunt-end single tube (BEST) protocol (Carøe et al., 2017). We then used quantitative PCR to determine the number of cycles required to reach an optimal amplification plateau for subsequent indexing PCR. After double index PCR, we verified successful library amplification using an Agilent Bioanalyzer instrument. In the case of control blanks, a Bioanalyzer results representing a "flatline' between size standards was considered a clean sample.

3.1.2. Extractions for vouchered contemporary & archival museum crocodile specimens.

All contemporary samples were provided in the form of aliquots of frozen whole blood (St. Augustine Alligator Farm Zoological Park-SAAF, AMCC) or as dried blood spots on Whatman filter paper (SAAF, MS and ERH). We extracted genomic DNAs using the Qiagen DNA Blood and tissue kit protocols for nucleated red blood according to methods provided by the supplier.

For the archival tissue samples from historical museum specimens, all sample manipulation took place in the dedicated clean DNA facilities at the AMNH as described in Hekkala et al. (2011). Small bits of tissue adhering to crania or postcranial material were collected from dried specimens with sterile tools and soaked in PBS for 12 h for rehydration. Between 30 and 90 mg of rehydrated tissue was then digested overnight at room temperature with Qiagen buffer ATL and Proteinase K and gentle shaking. All subsequent steps were according to the manufacturer's protocol with the exception that the final elution included heating of the EB buffer to 56C and letting the buffer rest on the column membrane for 15 min prior to centrifugation. Each sample was eluted twice in 80 μL (2 × 40 μL) buffer EB.

Genomic DNAs were shipped to Arbor Biosciences (Ann Arbor, MI, USA) in separate shipments for library preparation where specimen extracts were stored and handled separately. Samples were prepared as Illumina Truseq libraries prior to enrichment with either crocodilian DNA derived RNA baits (as in Enk 2014), or synthetic mitobaits derived from genomic resources (see 3.2 below). Libraries were created using 25 μL each of contemporary or archival DNA extract in Illumina® library preparations and index-amplified using unique P5 and P7 indexing primers (Meyer and Kircher, 2010) in 40 μL reactions using 10 μL of each library according to standard protocols. Amplifications were performed in real-time with a CFX96 Real-time PCR platform (BioRad). Indexed libraries were purified with MinElute to 15 μL TEB.

3.2. Genomic enrichment

3.2.1. Development of RNA baits derived from modern crocodilian DNA

Approximately 20 μL each of DNA extracts for six crocodilian taxa [*Crocodylus moreletii (n = 1), C. acutus (n = 1), C. siamensis (n = 1), C. suchus (n = 2), C. niloticus (n = 2) and Osteolaemus* tetraspis *(n = 3)*] from the AMCC at AMNH were shipped to Arbor Biosciences for global reverse transcription (both strands) with biotinylated rUTP using their proprietary procedure (Enk et al., 2014). This provided an aqueous suspension of approximately 100 μg mixed crocodilian RNA baits representing both intrageneric and outgroup taxa.

3.2.2. Enrichment of mummy samples

Genomic enrichment for the two replicated Kom Ombo mummy samples took place at the University of Copenhagen. Libraries for each mummy sample replicate (KomOmbo21 = replicate 1 and KomOmbo22 = replicate 2) and control blanks were enriched separately using both the whole genome (WGE) crocodilian derived bait set as described in Enk et al. (2014), and a targeted mitochondrial DNA (mitobaits) bait set (Arbor Biosciences 2017). For each enrichment, hybridizations were done at 48 °C for 48 h. Following bead cleanup and MinElute purification to 15 μL TEB, enriched eluates were amplified for 9 cycles and then purified with MinElute to 13 μL TEB. Each capture reaction used 1 μg of baits and 9 μL indexed library, which ranged from 0.2 to 5.8 ng/μL as estimated with total library quantification on a Hi-Sensitivity Bioanalyzer chip. Whole genome enriched and mito enriched libraries for each sample (KomOmbo21 or KomOmbo22) and blank libraries for each were pooled (50:50) and multiplexed with eight other unrelated ancient samples for sequencing on an Illumina HiSeq 4000 instrument at the Danish National High-Throughput Sequencing Centre at the University of Copenhagen.

3.2.3. Enrichment for vouchered contemporary and archival museum specimens

At Arbor Biosciences all contemporary and archival museum specimen DNAs were enriched separately using their custom MYbaits kit protocol. Each capture reaction used 1 μg of either crocodilian RNA baits or mitobaits and 9 μL indexed library, which ranged from 0.5 to 5.3 ng/μL as estimated with total library quantification on a Hi-Sensitivity Bioanalyzer chip. Hybridizations were done at 48 °C for 48 h. Following bead cleanup and MinElute purification to 15 μL TEB, enriched eluates were amplified for 10 cycles and then purified with MinElute to 13 μL TEB. Then 9 μL of these re-amplified enriched eluates were used in another round of capture using identical conditions as the first round except incubated at 55 °C for 39 h. These were cleaned and then purified with MinElute to 13 μL TEB, which we then re-amplified for 5 cycles. These final re-amplified doubly-enriched libraries were then purified to 13 μL TEB. Whole genome enriched (WGE) and targeted capture enriched (Mito) libraries were combined in pools in a 75/25 ratio and paired-end sequenced on one lane of an Illumina HiSeq® 2500 flow cell at the New York Genome Center.

4. Analytical methods

4.1. Mummy data

The adapters were initially removed using cutadapt v1.13 (Martin, 2011) and reads were aligned to mitochondrial reference sequences of *Crocodylus niloticus* (GB JF502243.1) and *Crocodylus suchus* (GB JF502244.1, an accession originally listed as *C. niloticus* in Genbank but currently recognized as *C. suchus*) using bowtie2 (Langmead and Salzberg, 2012) and then de-duplicated and filtered for minimum mapping quality q = 30 using SAMtools v1.4 (Li et al., 2009) (Table 2). Following mapping, we identified the most likely origin reference sequences according to sequence coverage and average coverage depth. We then took consensus sequences from these mapped reads of the mt

Table 2
Next Generation Sequencing (NGS) read statistics for enriched libraries for each crocodile mummy sample (KomOmbo21 or KomOmbo22) and the extraction blanks.

Sample name	Sample source	Raw reads	Deduplicated raw reads	Mapped to JF502243.1 Crocodylus niloticus	Mapped to JF502244.1 Crocodylus suchus	Endogenous %	Coverage depth JF502243.1	Coverage depth JF502244.1
KomOmbo21 (Rep 1)	Scapular surface tissue	10,053,918	7,010,213	4585	4591	0.065404573	12.3	12.3
KomOmbo22 (Rep 2)	Scapular bone	152,460,620	92,713,171	10,373	10,385	0.01118827	28.9	29
KomOmbo21 (Blank1)	NA	3101	2587	0	0	0	0	0
KomOmbo22 (Blank2)	NA	5035	4953	0	0	0	0	0

genome represented on the NCBI nucleotide (nt) database for both *C. niloticus* and *C. suchus*. Finally, we ran BLAST alignment on both consensus sequences against the entire nt database to confirm our identification.

In order to assess contamination during preparation we analyzed all blanks according to the same pipeline, and a subset of raw reads were checked for alignment to the NCBI nt database. To further assess authenticity of our assigned mitochondrial reads, we used the program mapDamage 2.0 (Jonsson et al., 2013) to assess patterns of aDNA strand length and C > U deamination patterns (Figs. 3 and 4).

4.2. Data from vouchered contemporary and crocodilian museum specimens

After sequencing, the reads from each sample were trimmed using the python script TQSfastq.py (http://genomics.pubs.princeton.edu/prv/resources/scripts/TQSfastq.py) with a quality (q) value of 20 and a minimum read length of 30. We then mapped the reads for each sample to our mitogenomic reference sequences using the program BWA-MEM with default settings (Li, 2013). After mapping, we identified and marked read duplicates using the tool MarkDuplicates from Picard (v. 1.77; http://broadinstitute.github.io/picard/). This was followed by indel realignment using IndelRealigner from the Genome Analysis Toolkit (GATK v.3.8; McKenna et al., 2010). Next, for each sample we used the program BCFtools (v. 1.9; Li, 2011) to call divergent sites with the 'mpileup' and 'call' commands. With 'mpileup' the maximum read depth was set to 1000. For the 'call' command, we used the multiallelic-caller with ploidy set to 1 (i.e. 'haploid'). For both commands, we included non-variant sites grouped into blocks by minimum depth (option '-g'). We then generated a consensus of each sample's mitochondrial genome incorporating divergent sites, while masking sites that had a read depth less than 10, a mapping quality less than 20, and/or a base quality less than 20. Masked sites were replaced with 'N's in the resulting FASTA file.

4.3. Phylogenetic analysis.

We examined the individually reconstructed mitochondrial genome sequences from the two replicated Salzkammergut mummy samples (KomOmbo21 and KomOmbo22) in a dataset including additional mitogenomes recovered from the newly sequenced contemporary and archival samples and published crocodilian mitogenomes from Genbank (Table 1). We included mt genome data for 12 extant species of *Crocodylus* and one outgroup (*Osteolaemus*). For *C. niloticus*, *C. suchus*, and other widespread members of genus *Crocodylus* we included multiple individual mt genomes to represent intraspecific variation. We aligned the mitochondrial genomes within this dataset using Clustal Omega (v. 2.1, Larkin et al., 2007). After alignment, we trimmed both ends of the sequence to match the sequence length of our Salzkammergut mummy samples.

With this aligned and trimmed dataset, we determined the best partitioning scheme and nucleotide substitution model for this data using PartitionFinder 2.1.1 (Lanfear et al., 2016) considering models implementable in RAxML (Stamatakis, 2014) and with small-sample-size corrected version of the Akaike Information Criterion (AICc) (Supplemental Table 1). The first, second, and third positions for the mitochondrial coding sequences were examined separately. Using the best partitioning scheme, we conducted both a maximum-likelihood (ML) and Bayesian Inference (BI) phylogenetic analyses (Darriba et al., 2012; Guindon and Gascuel, 2003). We conducted our ML phylogenetic analysis with RAxML 8.2.12 (Stamatakis, 2014). Measures of nodal support for ML analyses were generated from 1000 non-parametric bootstrap replicates and each run initiated from a random starting tree. MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004) was used to reconstruct phylogenetic relationships using BI. A Markov chain Monte Carlo process was set for four simultaneous chains with two million generations, each starting from a random tree and using the

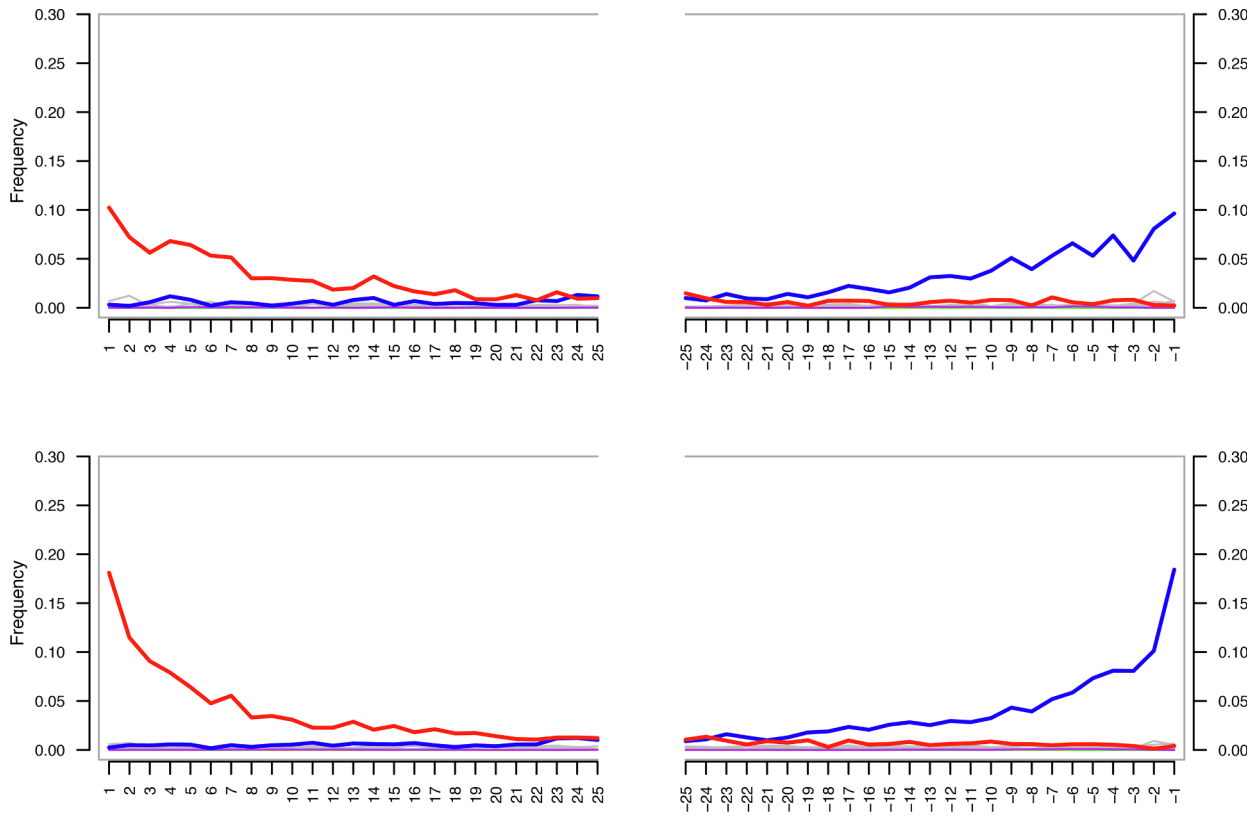

Fig. 3. MD: mapDamage deamination plot of sequencing reads mapped to the *Crocodylus suchus* mitochondrion, showing deamination patterns typical of ancient DNA. Upper panel A, KomOmbo21, replicate 1; lower panel B, KomOmbo22, replicate 2.

default heating scheme. Markov chains were sampled every 1000 generations, with the initial 25% of trees discarded as burn-in. Two million generations were sufficient for the standard deviation of split frequencies to decrease below 0.01.

5. Results

5.1. Sequencing results

Both replicates of ancient samples of the Salzkammergut crocodile mummy KomOmbo21 (tissue) and KomOmbo22 (bone) yielded adequate libraries for sequencing. Following trimming, deduplication, and mapping, our data suggested the most likely candidate references to both replicates to be the *C. suchus* published mitochondrial genome (JF502244.1), with coverage depth almost double the next best match *C. niloticus* (JF502243.1) (Table 2). Using the *C. suchus* mitochondrion as a proxy, we found that the normalized (i.e., having removed duplicate reads from the primary data) endogenous content of sample 21 (replicate 1) to be 0.07% and sample 22 (replicate 2) to be 0.01% (Table 2). Despite the endogenous DNA content of the samples being low, our coverage for the *C. suchus* mitogenome data was 99.9% refseq identity to the closest reference for both replicates, and between 12.3x and 29.0x mean coverage depth (Fig. 5). Due to lower copy number and generally low endogenous content, we were unable to recover nuclear gene regions in any depth from any sample libraries.

5.2. Sequence of authenticity and contamination

The fragment size distribution of mapped reads was typical of an ancient DNA assemblage, peaking at 42nt (Fig. 4). Cytosine deamination patterns were likewise typical of ancient DNA, exhibiting elevated C > U transitions at the terminal 5′ ends of sequencing reads, and symmetrically elevated G > A transitions and the 3′ ends (Fig. 3). We are therefore confident of the authenticity of these data. We ran a subset of reads through full metagenomics BLAST to a) perform secondary assessment endogenous content and b) assess potential contaminants. Although there was both microbial and human content in the raw read data, we were able to show that no contamination was present in any of the blanks from the extraction process by mapping the blank reads to two reference databases containing mitogenomes from human and crocodile. A single deduplicated sequence (n = 32) of the blanks contained in the reads mapped to crocodile mitochondrial database, and zero reads mapped to the human mitochondrion. A total of 205 reads from the Salzkammergut mummy samples mapped to the human mitochondrion; these were insufficient for authentication via mapDamage but few enough to be confident of no contamination from any source. The high content of human-derived reads in BLAST analysis, while not unexpected, could be from ancient human contamination from the mummification workshop or might also be explained as a function of database over-representation (Smith et al., 2015), a full analysis of which is beyond the scope of this study.

BLAST analysis of COX1 and D-loop regions showed that in all cases, the closest database hits by percent identity to the consensus sequences derived from *C. suchus*. The COX1 gene aligned to *C. suchus* in particular showed a significantly more supported match to the next-closest match (*C. niloticus*) at 4%, while the D-loop sequence was identical to our *C. suchus* reference D-loop with a 1% distance to the next match.

5.3. Phylogenetic analysis

We examined the position of each of the two reconstructed mitochondrial haplotypes from the Salzkammergut mummy in comparison with previously published complete mitogenomic data for five extant *Crocodylus* from GenBank and with newly recovered crocodilian mitogenomes for ten additional *Crocodylus* species representatives from modern samples and archival museum specimens in order to better

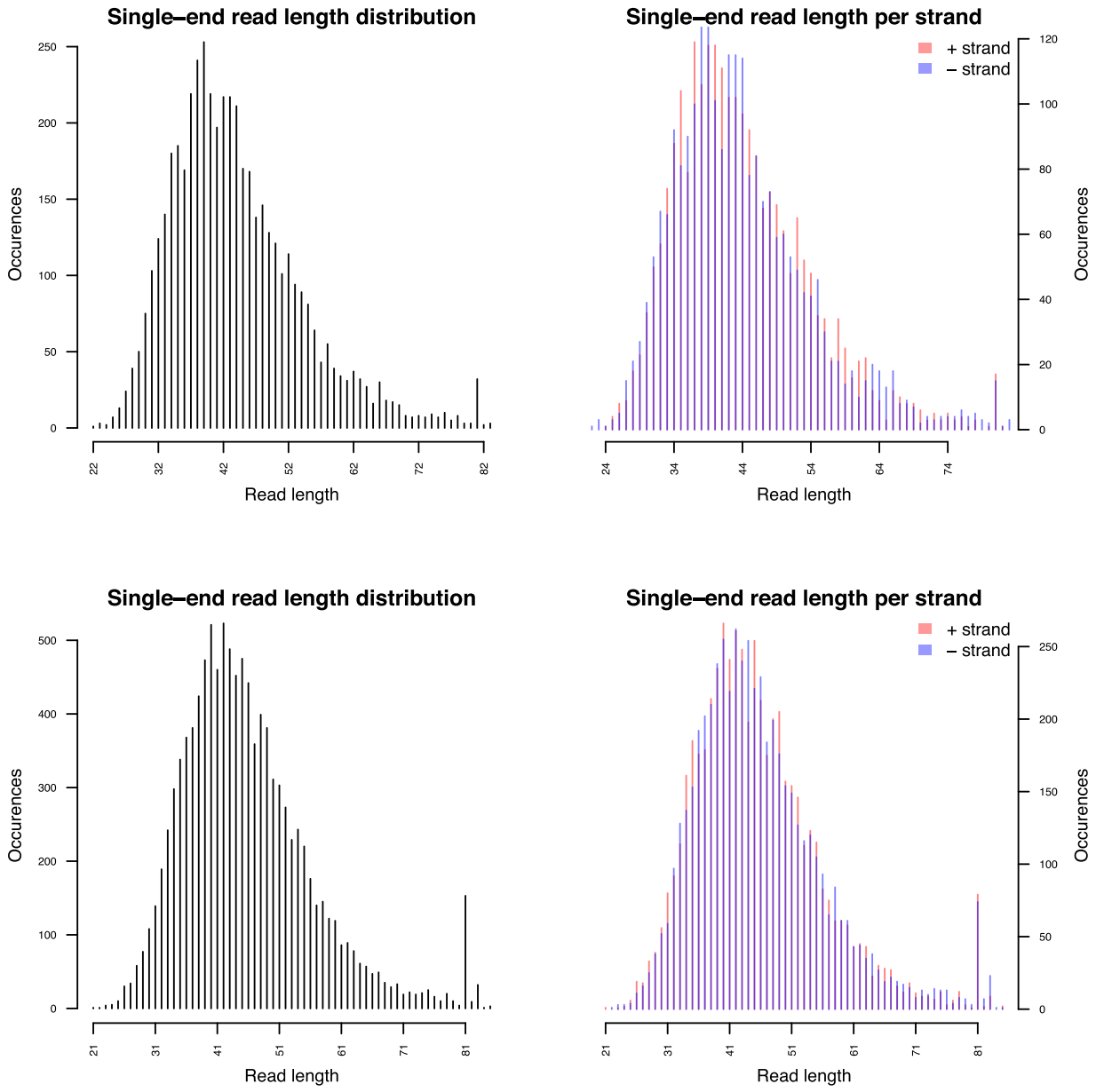

Fig. 4. FL: Fragment length plot of sequencing reads mapped to the *Crocodylus suchus* mitochondrion, showing fragment length distributions typical of ancient DNA. Upper panel A, KomOmbo21, replicate 1; lower panel B, KomOmbo22, replicate 2.

understand historical and ancient phylogeographic and phylogenetic variation within African *Crocodylus*. For our phylogenetic analyses, the best-fit model for each partition was the generalized time reversible model (Tavaré, 1986) with a proportion of invariable sites and a gamma distribution of rate heterogeneity (i.e. GTR + I + Γ model). See SI Table S1 for the complete details of partitions and models.

Both our Maximum Likelihood and Bayesian Inference analyses of aligned mitogenomes recovered from the crocodile mummy data resulted in nearly identical trees with no change in the placement of the mummy sequences (Fig. 6, Supplementary Fig. S1). For both tree-based placements, the consensus trees confirmed that the crocodile mummy from Salzkammergut represents *C. suchus*. Our results also indicate that haplotypes for two contemporary *C. niloticus* samples from modern Egypt form a distinct subclade within the clade containing all other contemporary and archival *C. niloticus* samples. We similarly found that five *C. niloticus* samples from Madagascar form a well supported subclade within *C. niloticus*.

6. Discussion

6.1. Ancient DNA from a mummy

The study of ancient DNA, particularly from anthropogenically mummified samples, has been fraught with issues of authenticity

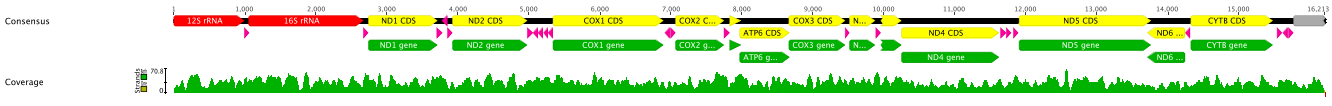

Fig. 5. Coverage plot of reads mapped to the *Crocodylus suchus* mitochondrion after duplicate removal and mapping filtering to minimum mapQ = 30.

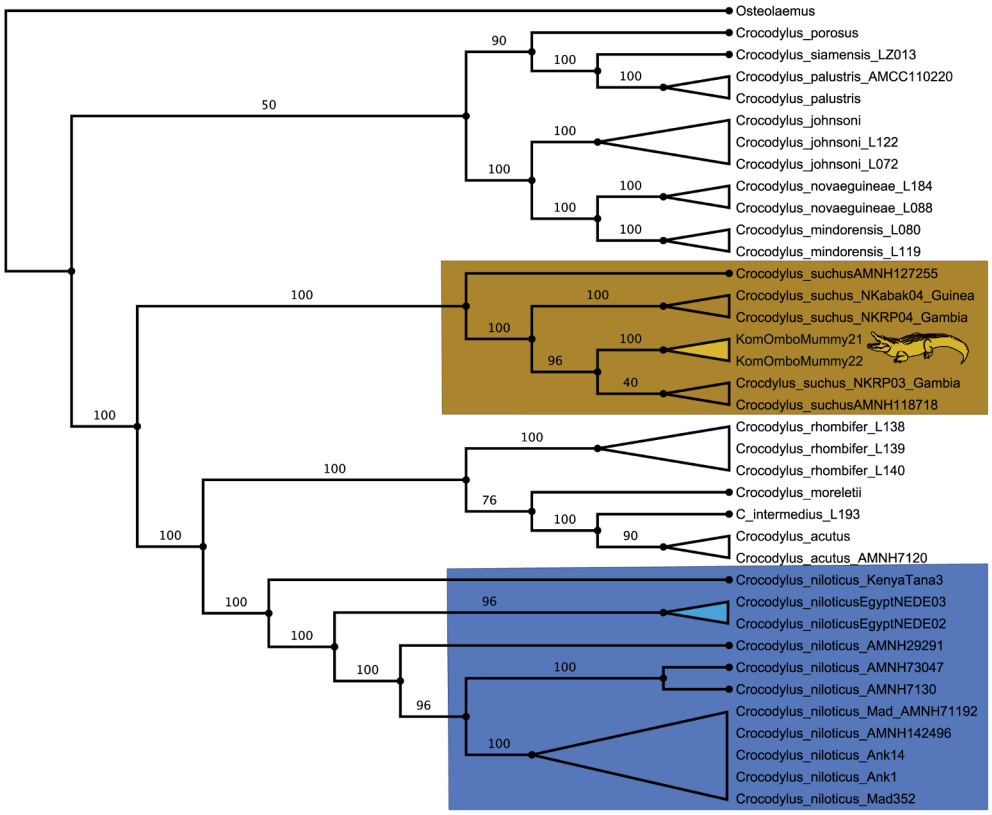

Fig. 6. Maximum likelihood cladogram representing the placement of partitioned mitochondrial genomes recovered from two replicated samples (KomOmbo21 and KomOmbo22) for the crocodile mummy from the Natural History Museum-G, Salzkammergut-Austria relative to extant members of Crocodilidae. Branch values are Maximum Likelihood bootstrap support. The placement confirms the mummy as *Crocodylus suchus*.

(Gilbert et al., 2005; Lorenzen and Willerslev, 2010). As in all aDNA analyses, multiple authentication methods must be applied to analyses of sequences obtained from ancient Egyptian mummified remains (Drosou et al., 2018). Recently tools have been developed to effectively differentiate time-dependent damage from naturally occurring mutations in the genome and to identify forms of microbial and modern contamination (Briggs et al., 2007). Our sequencing replicate results from duplicate samples derived from tissue and bone establish the presence of authentic mitogenomic data from an individual crocodile mummy. Further, they highlight the potential for sequencing enriched DNA libraries to recover nearly complete mitogenomes from crocodile mummies dating to ca. 3rd century BC − 2nd century AD, the acme of animal mummification, and the time when most Sobek temples associated with mummies were active (Bresciani, 2015).

6.2. Taxonomic identity and phylogeographic patterns

In his 1807 description of the sacred crocodile, *C. suchus,* Geoffroy Saint-Hilaire referred extensively to earlier anecdotal evidence from Herodotus's Histories, elements of which also appear in Strabo's 1st century BCE account on Egypt (Book XVII: 38, 39, 47). According to Herodotus, in ancient Egypt a type of sacred crocodile was cared for in temples and adorned with golden earrings and bracelets. These sacred crocodiles were fed on cakes and wine mixed with honey, mummified, and placed in temples (Herodotus II: 69). The Egyptian sites where crocodile deities were most common were the Fayum in the north, and Thebes and Kom Ombo in the south, with a few other sites, such as Ma'abdeh, in between.

Our phylogenetic analyses of independently replicated mitogenomes from two types of tissue from the crocodile mummy from the Salzkammergut confirm this adult male specimen to be *C. suchus,* "the sacred crocodile". Geoffroy Saint-Hilaire (1807) cited both behavioral and cultural interpretations of what Egyptians considered to be crocodilian "taxa" and combined those with his own morphological assessment of species' differences in his description of *C. suchus*.

The mummification of this adult male in the style of the temple crocodile mummies of Kom Ombo, suggests that the choice of this particular species, rather than *C. niloticus*, was indicative of a specific cultural role for it in the worship of the crocodile god Sobek. Egyptians may have preferred *C. suchus* over *C. niloticus* for this role as the former are typically smaller and less aggressive to humans (Brito et al., 2011). Similar descriptions of "tame" crocodiles found in the Ennedi Plateau in Chad (Klemens, pers comm.) turned out to be *C. suchus* as well (Schmitz et al., 2003). Even today throughout West Africa, traditional beliefs in Gambia (Moiser and Barber, 1994), Benin (Kpera, 2003), Burkina Faso (Toonen, 2003), and Mauritania (Brito et al., 2011, Velo-Antón et al., 2014), among others, hold that these sacred crocodiles will not attack swimmers and will protect those who revere them.

The persistence of isolated populations identified as *C. suchus* in the Sahara of Mauritania and central Chad, as well as extensive pictographic evidence for crocodiles throughout the Sahara (A. Zboray, 2017 pers comm.; de Smet, 1998), and previous research confirming *C. suchus'* presence along with *C. niloticus* in the White Nile of Sudan up until 1922 (Hekkala et al., 2011), all suggest a prior contiguous distribution of this species across Saharan Africa. The potential for an ancient northern African distribution of *C. suchus* with a relatively recent contraction is also supported by projections of the distribution of wetter habitats during the mid-Holocene (Egerer et al., 2018; Nicolas et al., 2018). Regular connectivity between the Nile and ancient lake Mega-Chad, and into the Western Nile River Basin likely facilitated the distribution of many species across the Sahara, including humans (Drake et al., 2011), varanids (Dowell and Hekkala, 2016; Dowell et al., 2016), and bufonids (Nicolas et al., 2018). Paleoclimatic records during the mid-Holocene African Humid Period (6–8000 ybp) (Brito et al., 2014; Egerer et al., 2018) and ecological niche modeling (Cunningham 2015) support a historically more favorable climate for continuous distribution of these taxa than is found today. This evidence and data from recent surveys and genetic analyses strongly suggest that the current distribution of *C. suchus* is much reduced relative to its prior distribution across Sahara and within the Nile River drainage (Fig. 2).

Inference of species' distributions based on samples from archaeological contexts are, by their nature, limited as humans have a long history of moving animals around for both cultural and agricultural purposes. Previous data from mummies of hatchling *C. suchus* in Thebes (Hekkala et al., 2011) is equivocal regarding the presence of wild *C. suchus* in the Egyptian Nile. It is possible that crocodiles, or even unhatched eggs, were brought from other locales or were obtained via captive breeding in local temples (Molcho, 2014). Local sourcing of adult crocodiles for mummification is also a possibility. A recent forensic examination of the stomach contents and cause of death of a slightly smaller mummified crocodile from Kom Ombo indicates that it was hunted from the wild (Porcier et al., 2019). In the absence of tangible evidence regarding ecological and habitat partitioning between *C. suchus* and *C. niloticus*, a better understanding of the habitat variability in the Egyptian Nile watershed during this time period would at least facilitate hypotheses about the two species' specific distributions.

Although confirmation of whether adult or hatchling *C. suchus* individuals were native to the Egyptian Nile or imported for use in temples will require additional sampling from both paleontological and bioarchaeological contexts, the mitogenomic data from a crocodile mummy presented here contributes to our understanding of human animal interactions within the context of historical biogeography and climate change. Genomic analyses of crocodilian samples from earlier paleontological contexts in the region of the Fayum and other Egyptian sites could further elucidate the relative influences of recent climate change versus shifting anthropogenic cultural practices over recent millennia on the distribution of this species in Egypt.

7. Conclusions

As Geoffroy Saint-Hilaire proposed two centuries ago, animal mummies may provide a framework for understanding how species respond to changing environmental conditions. New methods of sequence capture and high throughput sequencing using next-generation sequencing (NGS) technologies now allow for the reconstruction of ancient mitogenomes from extinct populations of wild animals and from bioarchaeological materials (Vilstrup et al., 2013). Recently ancient genomic and bioinformatic methods have allowed for the empirical demonstration of changes to the genomes and distributions of wild animal populations as humans have transformed the global landscape (Johnson and Munshi-South, 2017).

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Author contributions

EH, SI, OS, and MTPG conceived the study. EH and OS conducted lab work and analyses and wrote the manuscript. MHS and KAV provided samples and contributed to manuscript writing and development. SWC contributed distributional data and niche modeling background for *C. suchus*. MA and AN conducted data analyses and wrote portions of the manuscript. SI conducted specimen research and wrote portions of the manuscript. GA and MTPG provided laboratory and logistical support and contributed editorial support to the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jasrep.2020.102483.

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