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Mitochondrial DNA Phylogeography of *Caiman crocodilus* in Mesoamerica and South America

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ABSTRACT The Neotropical crocodylian species, *Caiman crocodilus*, is widely distributed through Mesoamerica, northern South America, and the Amazon basin. Four subspecies are recognized within *C. crocodilus*, suggesting some geographic variation in morphology. In this study, we utilized mitochondrial DNA (mtDNA) sequence data from 45 individuals of *C. crocodilus* throughout its range to infer its evolutionary history and population structure, as well as to evaluate genealogical support for subspecies and their geographic distributions. Our molecular phylogenetic results identified five mtDNA haplotype clades with a mean sequence divergence of 3.4%, indicating considerable evolutionary independence among phylogeographic lineages. Our results were also broadly consistent with current subspecific taxonomy, with some important additional findings. First, we found substantial genetic structuring within *C. c. fuscus* from southern Mesoamerica. Second, though we confirmed the existence of a widespread Amazonian clade, we also discovered a cryptic and divergent mtDNA lineage that was indistinguishable from *C. c. crocodilus* based on external morphology. Third, we confirm the status of *C. c. chiapasius* as a distinct evolutionary lineage, and provide evidence that *C. c. fuscus* may be moving northward and hybridizing with *C. c. chiapasius* in northern Mesoamerica. Finally, our results parallel previous phylogeographic studies of other organisms that have demonstrated significant genetic structure over shorter geographic distances in Mesoamerica compared with Amazonia. We support conservation efforts for all five independent lineages within *C. crocodilus*, and highlight the subspecies *C. c. chiapasius* as a unit of particular conservation concern. *J. Exp. Zool. 309A:614–627, 2008.*

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One goal of conservation biology lies in preserving the natural diversity of independent evolutionary lineages on earth (Primack, 2002). For species of particular concern, such as certain charismatic or economically important tetrapods, biologists and the public are also interested in preserving distinct lineages below the species level, especially in the case of named subspecies (Birstein et al., '98). The growing challenge confronting conservationist biologists is how best to apply limited resources to aid an expanding roster of endangered species and subspecies (Avise, '89; Amato and Gatesy, '94).

The spectacled caiman, *Caiman crocodilus* Linnaeus 1758, is a widespread Neotropical crocodylian of special conservation concern owing to locally intensive exploitation of these animals as a source of valuable hides (MacGregor, 2002). Four subspecies are recognized based on morphological differences although the evolutionary

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distinctiveness of the subspecies has not been previously assessed. In the worldwide trade in crocodylian skins, 70% of all skins come from Neotropical animals and the vast majority of these skins are taken from *C. crocodilus*. This species is also trafficked through the pet trade but in relatively minor quantities compared with the skin trade. *C. crocodilus* is more frequently ''farmed'' than any other crocodylian species in Latin America. However, most harvesting and captive breeding programs ignore the evolutionary and ecological distinctiveness that may underlie the subspecific taxonomy.

Characterizing intraspecific population structure allows wildlife managers to assign unknown individuals to their geographical source population, thereby helping captive breeding programs and farms avoid outcrossing of distinct lineages (Densmore and Ray, 2001; MacGregor, 2002), as well as improving the efficiency of reintroduction programs (Densmore and Ray, 2001; Venegas-Anaya, 2001). Effective, long-term conservation of *C. crocodilus* will therefore benefit significantly from the identification of unique intraspecific evolutionary lineages.

Mitchondrial DNA provides the most efficient marker available for characterizing the geographic population structure of a species for which other genetic markers have not yet been developed (Brown, '79; Cann and Wilson, '83; Avise et al., '84, '87; Avise, '94; Bermingham and Avise, '86). Although mtDNA provides only a single evolutionary genetic marker that may not be representative of the variability present across the nuclear genome (Hoelzer, '97; Densmore and Ray, 2001), and though independent markers may give contrasting phylogenetic signal (Takahata, '89; Beltrán et al., 2002), mtDNA is particularly useful in identifying and delineating distinct evolutionary lineages and inferring their relationships (Moore, '95,'97). MtDNA genotyping also provides direct benefits to conservation biology. Many wild caiman are illegally harvested, and mtDNA phylogeographic data can provide an economical tool for law enforcement to identify the geographic source of contraband animals or skins (Thorbjarnarson, '92; Ross, '98).

Given its wide geographic range, *C. crocodilus* also provides an excellent model to study the influence of geological and environmental history on the origin and distribution of species in the Neotropics. The mtDNA phylogeography of *C. crocodilus* permits biogeographic assessment of how geological and climatological events such as the closure of the Panama Isthmus, the uplift of the northern Andes, sea level changes, or habitat fluctuations during Pleistocene glaciation events (Haffer, '82; Haq et al., '87; Colinvaux et al., '96; Gregory-Wodzicki, 2000; Coates et al., 2004; Kirby and MacFadden, 2005) have influenced the diversification of lineages of *C. crocodilus* (Bermingham and Avise , '86; Avise et al.,). We can then evaluate biogeographic hypotheses concerning the origins of Neotropical diversity by comparing our findings from *C. crocodilus* with other species (Bermingham and Martin, '98; Slade and Moritz, '98; Perdices et al., 2002; Cortés-Ortiz et al., 2003; Eberhard and Bermingham, 2005; Cheviron et al., 2005; Patton and Da Silva, 2005; Wüster et al., 2005; Camargo et al., 2006; Crawford et al., 2008; Wang et al., submitted).

The goals of this study were to characterize the genetic variation within *C. crocodilus*, to test the validity of currently recognized subspecies and their distributions and to infer the evolutionary history of this important species. We obtained mtDNA sequence data from across the species' range, including three of the four subspecies of *C. crocodilus*. We also compared our data with those of Vasconcelos et al. (2006) for Amazonian *C. crocodilus*. We found that the mtDNA phylogeny of *C. crocodilus* samples is compatible with current subspecific taxonomy, but this taxonomy obscures additional mtDNA lineages in southern Mesoamerica and Amazonia.

METHODS

Sampling

The systematics of the genus *Caiman* remains somewhat contentious, but the most accepted taxonomy divides the lineage into three species: *C. crocodilus*, *C. yacare*, and *C. latirrostris* (King and Burke, '89; Busack and Pandya, 2001). All three species are Neotropical lowland inhabitants, with a maximum elevation of 400 m. The latter two species are South American endemics, whereas *C. crocodilus* ranges from southern Mexico to northern South America, including the Amazon River basin. Based on geographic, phylogenetic, and fossil evidence, *C. crocodilus* is thought to have a South American origin (Vanzolini and Heyer, '85; Brochu, 2000, 2004; Aguilera et al., 2006; Martin, 2007).

We collected a total of 45 samples of *C. crocodilus* from across its range. According to the most widely accepted subspecific taxonomy for *C. crocodilus* (King and Burke, '89; Rodríguez,

2000; Busack and Pandya, 2001; Vasconcelos et al., 2006; Escobedo et al., 2008), we collected the following samples per subspecies (see Table 1 and Fig. 1 for details). *C. c. crocodilus* Linnaeus 1758 is distributed across the Amazon River basin, and we collected a total of 11 samples of this subspecies from northern and central Amazonian Peru. *C. c. fuscus* Cope 1868 ranges throughout southern Mesoamerica and both sides of the northern Andes of South America, and we collected 31 samples from Costa Rica, Panama, and the Caribbean coast of Colombias. *C. c. chiapasius* Bourcurt 1876 is restricted to northern Mesoamerica, and we collected three samples from the Pacific coast of Mexico. *C. c. apoporensis* Medem 1955 is endemic to the Apoporis River of *cis*-Andean Colombia, and samples of this subspecies were unavailable. We also included and re-analyzed the mtDNA data of Vasconcelos et al. (2006) consisting of 38 cytochrome *b* (Cyt *b*) haplotypes for *C. c. crocodilus* obtained from across the Amazon basin.

Field methods

Individuals of *C. crocodilus* were easily identified to subspecies in the field based on the following external characters: body size, head size, general coloration, inter-ocular distance, relationship between inter-ocular distance, and the distance from the infra orbital bridge to the snout (Medem, '81,'83; Busack and Pandya, 2001). Samples were taken arbitrarily with respect to sex and size of the animal. From each individual sampled, one scale was clipped from the tail and preserved in DMSO/EDTA buffer (Seutin et al., '91) or in 95% ethanol. We sampled a total of 45 individuals of *C. crocodilus* and two outgroup species: *Alligator mississippiensis* and *Paleosuchus trigonatus*. Samples were analyzed and stored at the Molecular Evolution Laboratory of the Smithsonian Tropical Research Institute (STRI), Republic of Panama.

Laboratory methods

From each scale clipping, genomic DNA was isolated by proteinase K digestion and extracted using the CTAB/phenol/chloroform technique (Sambrook et al.,'89; Palumbi et al., '96). The entire Cyt *b* gene was amplified by PCR in two overlapping pieces using two primer pairs: L14211 (5′-AAG ATC TGA ARA ACC YCG TTG-3′) (Venegas-Anaya, 2001) with CB3H (5′-GGC AAA TAG GAA RTA TCA -3′) (Palumbi, '96), and L14849 (5′- TCC TCC ACG AAC GCG GAR C-3′) with H15453 (5′-CCK TCC AYY TCT GTC TTA CAA G -3′) (Venegas-Anaya, 2001). We also amplified a 658 basepair (bp) fragment from the 3′ half of the cytochrome oxidase I (COI) gene using the primer pair COIa (5′-AGT ATA AGC GTC TGG GTA GTC -3′) with COIf (5′-CCT GCA GGA GGA GGA GAY CC -3′) (Kessing et al., '89).

For both the genes, double-stranded DNA was amplified in 25 μL reactions: 2.5 μL of 10 μM Tris-HCl buffer, 1.25 μL of 2.0 μM $MgCl_2$, 1.25 μL of 10 μM of each primer, 2.5 μL of dNTP containing 200 μM of each nucleotide, 15.05 μL of ddH_20, 1 μL of template DNA, and 0.20 μL (1UI) Amplitaq polymerase (Qiagen, Valencia, CA.). The following thermocycler program was used: initial denaturation at 94°C for 120 sec, denaturation at 94°C for 45 sec, annealing at 53°C for 45 sec, extension at 72°C for 90 sec, repeated for 5 cycles, followed by 29 cycles with annealing at 58°C. The PCR products were electrophoresed in 1.5% low melting point agarose gels using a Tris-acetate buffer (pH 7.8) containing 1 μg/mL of ethidium bromide. Three μL of a gel-purified PCR product were used as template in a 10 μL cycle sequencing reaction using a BigDye 3.1 terminator cycle sequencing kit (Applied Biosystems, Forester City, CA.). Each PCR product was sequenced in both directions using the same primers used for the PCR amplification. After cycle sequencing, samples were run on an ABI 3100 capillary sequencer (Applied Biosystems, Forester City, CA.) following manufacturer's protocol. Chromatograms were reviewed, assembled, and aligned using Sequencher version 4.5 (Gene Codes, Ann Arbor, MI). DNA sequences were translated into amino acids and reviewed in MacClade version 4.1 (Maddison and Maddison, 2005). All DNA sequences were in GenBank (Table 1).

Analytical methods

Congruence between the Cyt *b* and the COI data sets was evaluated using the partition homogeneity test (Mickevich and Farris, '81; Farris et al., '94) as implemented in PAUP* version 4.0b10 (Swofford, 2003) and using 1,000 permutations of the combined data set. Nucleotide composition of the Cyt *b* and COI genes was examined with the software Sequencer version 6.1 (Kessing, 2000), and a χ^2 test for heterogeneity in nucleotide frequencies was performed with PAUP*.

Phenetic analysis was performed using the neighbor-joining algorithm (BioNJ) (Saitou and

TABLE 1. Samples of Caiman crocodilus plus two outgroups used in this study

Voucher number	Locality	Longitude	Latitude	Country	Haplotype	Subspecies	GenBank COI	GenBank Cyt b
CAcrfu13RSJuanCRATL	Río San Juan	−84.7833	10.8667	CR	I	*C.c. fuscus*	EU26016	EU496817
CAcrfu31RSJuanCRATL	Río San Juan	−84.7833	10.8667	CR	II	*C.c. fuscus*	EU26017	EU496818
CAcrfu41RSJuanCRATL	Río San Juan	−84.7833	10.8667	CR	III	*C.c. fuscus*	EU26018	EU496819
CAcrfu42RSarapiquiCRATL	Río Sarapiquí	−84	10.5	CR	II	*C.c. fuscus*	EU26019	EU496820
CAcrfu44RSarapiquiCRATL	Río Sarapiquí	−84	10.5	CR	II	*C.c. fuscus*	EU26020	EU496821
CAcrfu46RCSuciaSVPAC	RíoCara Sucia	−89.9409	13.7618	SV	IV	*C.c. fuscus*	EU26021	EU496822
CAcrfu47RCSuciaSVPAC	Río Cara Sucia	−89.9409	13.7618	SV	IV	*C.c. fuscus*	EU26022	EU496823
CAcrfu48RCSuiaSVPAC	Río Cara Sucia	−89.9409	13.7618	SV	IV	*C.c. fuscus*	EU26023	EU496824
CAcrfu49SSalvadorSVPA	San Salvador	−88.6963	13.2439	SV	V	*C.c. fuscus*	EU26024	EU496825
CAcrfu51SSalvadorSVPA	San Salvador	−88.6963	13.2439	SV	VI	*C.c. fuscus*	EU26025	EU496826
CAcrfu53SSalvadorSVPA	San Salvador	−88.6963	13.2439	SV	V	*C.c. fuscus*	EU26026	EU496827
CAcrfu55SSalvadorSVPA	San Salvador	−88.6963	13.2439	SV	V	*C.c. fuscus*	EU26027	EU496828
CAcrfu59RParritaCRPAC	Río Parrita	−84.3333	9.5	CR	VII	*C.c. fuscus*	EU26028	EU496829
CAcrfu62RParritaCRPAC	Río Parrita	−84.3333	9.5	CR	IX	*C.c. fuscus*	EU26029	EU496830
CAcrfu66RParitaCRPAC	Río Parrita	−84.3333	9.5	CR	VII	*C.c. fuscus*	EU26030	EU496831
CAcrfu69RParritaCRPAC	Río Parrita	−84.3333	9.5	CR	X	*C.c. fuscus*	EU26031	EU496832
CAcrfu70RArmilaPAATL	Río Armila	−77.4167	8.66667	PA	XI	*C.c. fuscus*	EU26032	EU496833
CAcrfu72RArmilaPAATL	Río Armila	−77.4167	8.66667	PA	XI	*C.c. fuscus*	EU26033	EU496834
CAcrfu74RArmilaPAATL	Río Armila	−77.4167	8.66667	PA	XII	*C.c. fuscus*	EU26034	EU496835
CAcrfu79RMagdalenaCOATL	Río Magdalena	−74.85	11.1	CO	XIII	*C.c. fuscus*	EU26035	EU496836
CAcrfu83RTuiraPAPAC	Río Tuira	−78.08	8.55	PA	XIV	*C.c. fuscus*	EU26036	EU496837
CAcrfu85RSanSanPAATL	Río SanSan	−82.5167	9.51667	PA	XV	*C.c. fuscus*	EU26037	EU496838
CAcrfu86RSanSanPAATL	Río SanSan	−82.5167	9.51667	PA	XVI	*C.c. fuscus*	EU26038	EU496839
CAcrfu90RBalsaPAPAC	Río Balsa	−77.99	8.2	PA	XVII	*C.c. fuscus*	EU26039	EU496840
CAcrfu95RLaMaestraPAPAC	Río La Maestra	−78.73	8.88	PA	VII	*C.c. fuscus*	EU26040	EU496841
CAcrfu106SJuanPAPAC	Río San Junan	−81.98	8.25	PA	XVIII	*C.c. fuscus*	EU26041	EU496842
CAcrfu108CulebraPAATL	Canal de Panama	−79.2	9.53333	PA	VIII	*C.c. fuscus*	EU26042	EU496843
CAcrfu109CulebraPAATL	Canal de Panama	−79.2	9.53333	PA	XIX	*C.c. fuscus*	EU26043	EU496844
CAcrch120TapachulaMXATL	Tapachula	−92.2833	14.9	MX	XX	*C.c. chiapasius*	EU26044	EU496845
CAcrch122TapachulaMXATL	Tapachula	−92.2833	14.9	MX	XXI	*C.c. chiapasius*	EU26045	EU496846
CAcrch123TapachulaMXATL	Tapachula	−92.2833	14.9	MX	XX	*C.c. chiapasius*	EU26046	EU496847
CAcrfu139RChagresPAATL	Río Chagres	−79.95	9.28	PA	XXII	*C.c. fuscus*	EU26047	EU496848
CAcrfu141RSJuanPAPAC	Río San Juan	−81.98	8.25	PA	XXIII	*C.c. fuscus*	EU26048	EU496849
CAcrfu142RSJuanPAPAC	Río San Juan	−81.98	8.25	PA	XXIII	*C.c. fuscus*	EU26049	EU496850
CAcrcr178AmazonasPEATL	Rio Istocohca	−75	−5	PE	XXIV	*C.c. crocodilus*	EU26050	EU496851
CAcrcr179AmazonasPEATL	Rio Istocohca	−75	−5	PE	XXV	*C.c. crocodilus*	EU26051	EU496852
CAcrcr251RUcayaliPEATL	Río Ucayalí	−74.5381	−8.3825	PE	XXVI	*C.c. crocodilus*	EU26052	EU496853
CAcrcr255RUcayaliPEATL	Río Ucayalí	−74.5381	−8.3825	PE	XXVI	*C.c. crocodilus*	EU26053	EU496854
CAcrcr260RUcayaliPEATL	Río Ucayalí	−74.5381	−8.3825	PE	XXVII	*C.c. crocodilus*	EU26054	EU496855
CAcrcr265RUcayaliPEATL	Río Ucayalí	−74.5381	−8.3825	PE	XXVII	*C.c. crocodilus*	EU26055	EU496856
CAcrcr279RIstocohcaPEATL	Río Amazonas	−75	−5	PE	XXVIII	*C.c. crocodilus*	EU26056	EU496857
CAcrcr281RIstocohcaPEATL	Rio Istocohca	−75	−5	PE	XXIX	*C.c. crocodilus*	EU26057	EU496858
CAcrcr282RIstocohcaPEATL	Rio Istocohca	−75	−5	−PE	XXVII	*C.c. crocodilus*	EU26058	EU496859

TABLE 1. Continued

Voucher number	Locality	Longitude	Latitude	Country	Haplotype	Subspecies	GenBank COI	GenBank Cyt b
CAcrcr283RIstocohcaPEATL	Río Istocohca	−75	−5	PE	XXX	*C.c. crocodilus*	EU26059	EU496860
CAcrcr284RIstocohcaPEATL	Río Istocohca	−75	−5	PE	XXXI	*C.c. crocodilus*	EU26060	EU496861
PAtr169RUcayaliPEATL	Río Ucayalí	−74.5381	−8.3825	PE		*Paleosuchus trigonatus*	EU26061	EU496862
ALmi111USA				US		*Alligator mississippiensis*	EU26062	EU496863

The first six letters of the voucher number refer to the genus, species, and subspecies of the sample. Unique haplotypes of *C. crocodilus* are arbitrarily numbered, and these numbers appear in Fig. 2. Subspecific designations are based on morphological observations taken in the field.

Nei, '87; Gascuel, '97) and the caiman phylogeny was inferred using the maximum likelihood (ML) criterion (Felsenstein, '81, 2004) as implemented in PAUP*. We used heuristic searches with TBR branch swapping in the ML analyses. Bayesian MCMC phylogenetic inference (Rannala and Yang, '96; Yang and Rannala, '97) was implemented using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) for Macintosh. We analyzed the combined DNA sequence data using two approaches to data partitioning: a two-partition analysis by gene and a three-partition analysis by codon position. For each Bayesian analysis we ran parallel MCMCs with eight metropolis-coupled chains each for five million generations, sampling trees every 1,000 generations, and gauging convergence by the split frequencies between parallel runs and by visualization of the burn-in of the −Ln scores. Sampled trees from both runs obtained after the burn-in period were used to construct a 50% majority rule consensus tree in which marginal posterior probabilities of each clade were indicated by the clade's proportional representation in the posterior distribution of trees. We considered clade probabilities of 95% or greater as significant. For ML and partitioned Bayesian analyses, we selected the best-fit models of DNA sequence evolution for the combined data using Modeltest version 3.7 (Posada and Crandall, '98) and for each data partition using MrModeltest version 2.2 (Nylander, 2004). Clade support was also evaluated using the nonparametric bootstrap (Felsenstein, '85), with each pseudo-replicate data set analyzed by the NJ method. For all phylogenetic analyses, *A. mississipiensis* was assigned as the outgroup taxon, following Brochu (2000). The congruence between topologies of NJ, ML, and Bayesian consensus trees was tested using the Shimodaira–Hasegawa test as implemented in PAUP* with 1,000 bootstrap replicates (Shimodaira and Hasegawa, '99; Shimodaira, 2001, 2002).

We conducted a second ML phylogenetic analysis using only Cyt *b* data in which we combined our data with all unique haplotypes reported by Vasconcelos et al. (2006) in their study of Amazonian *C. crocodilus*. Both studies included samples from Amazonian Peru, while Vasconcelos et al. (2006) also covered the Brazilian Amazon, allowing us to extend the geographic range of our analysis of *C. crocodilus* population structure.

In order to estimate divergence times among species and subspecies from the combined mtDNA data set, we first tested whether the data conformed to a clock-like model of evolution using

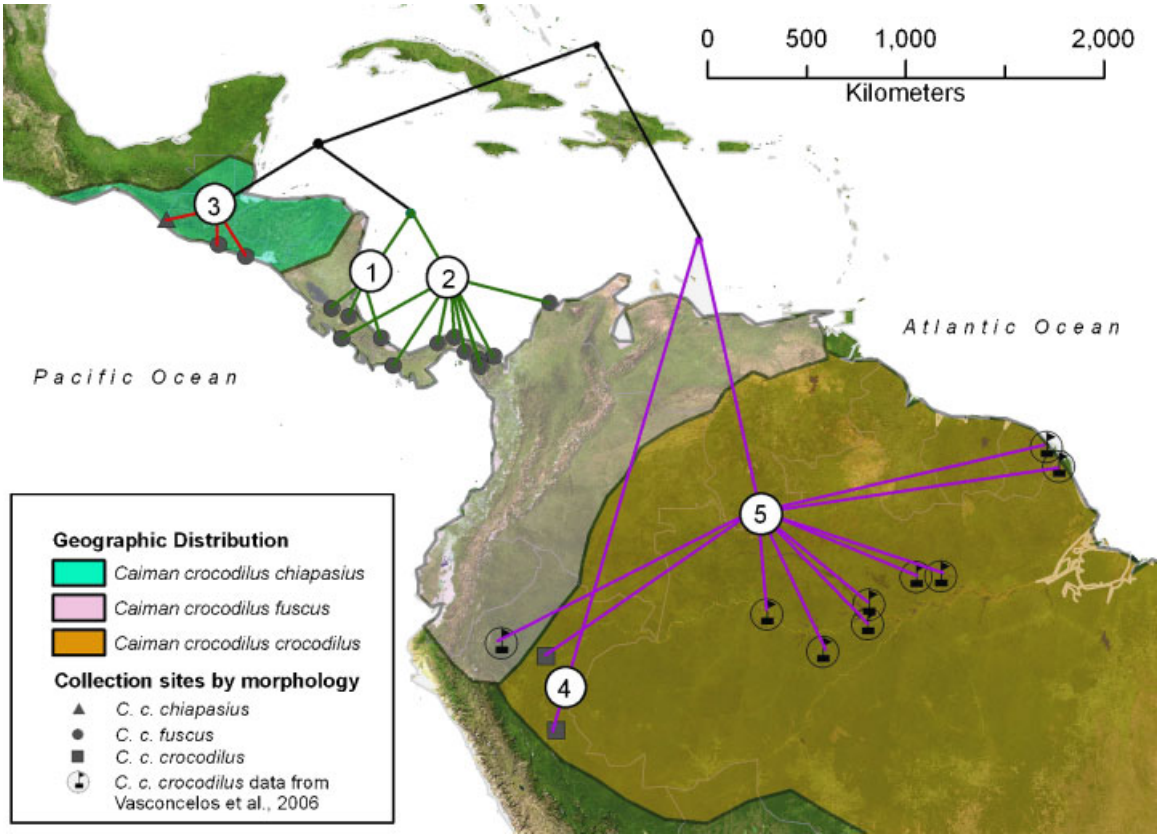

Fig. 1. Map of tropical America showing the traditionally accepted geographic ranges of three subspecies of *Caiman crocodilus* as well as collection sites for samples used in this study. Sampling localities are connected by a diagrammatic cladogram based on the maximum likelihood topology (Fig. 2) of 31 unique mtDNA haplotypes obtained here plus the Cyt *b* haplotypes reported for Amazonian *C. crocodilus* by Vasconcelos et al. (2006). The five main clades of *C. crocodilus* are numbered here the same as in Figure 2. Locality symbols indicate subspecies as determined by morphology of the animal, not by geographic source of the sample. For example, from within the historical distribution of *C. c. chiapasius* we collected ten samples from three sites, of which the three samples from the northernmost site were identified by morphology as *C. c. chiapasius* and the other seven more southern samples were identified as *C. c. fuscus* (Table 1). Note, the samples of Vasconcelos et al. (2006) were not identified to subspecies. Cyt *b,* cytochrome *b*.

a log-likelihood ratio test of the ML tree vs. a ML clock-enforced tree (Felsenstein, '81; Page and Holmes, '98). We then used published fossil data to calibrate the ages of the nodes on our ML tree. The age of the most recent common ancestor (MRCA) of *Alligator* and *Caiman*, the root of our molecular phylogeny, has been estimated at 60–65 million years ago (mya) (Densmore, '83; Brochu, 2000), 65–70 mya (Estes and Báez, '85), and 65–72 mya (Roos et al., 2007). Therefore, we alternatively assumed calibration times of 60 or 70 mya for the root node to obtain a range of evolutionary rates of model-corrected mtDNA sequence divergence for our phylogeny.

RESULTS

All 1,236 bp of the Cyt *b* gene and a 657 bp fragment of the COI gene were sequenced from 45 *C. crocodilus* individuals, one *P. trigonatus*, and one *A. mississippiensis* (Table 1). Among *C. crocodilus* samples, Mesoamerica is represented by 33 samples and South America by 12 samples.

Molecular characterization of mitochondrial Cyt b and COI genes

For both the genes, the majority of variable and informative sites were found in the third codon position. Base frequencies were homogeneous across taxa ($P = 1.0$ for both the genes). No gene sequences exhibited premature stop codons when translated into amino acid sequences. From the 45 *C. crocodilus* individuals sampled, we obtained 31 unique haplotypes (Table 1). Among all 1,894 characters 1,380 were constant, 307 were parsimony uninformative, and 207 were parsimony informative. Cyt *b* and COI showed similar

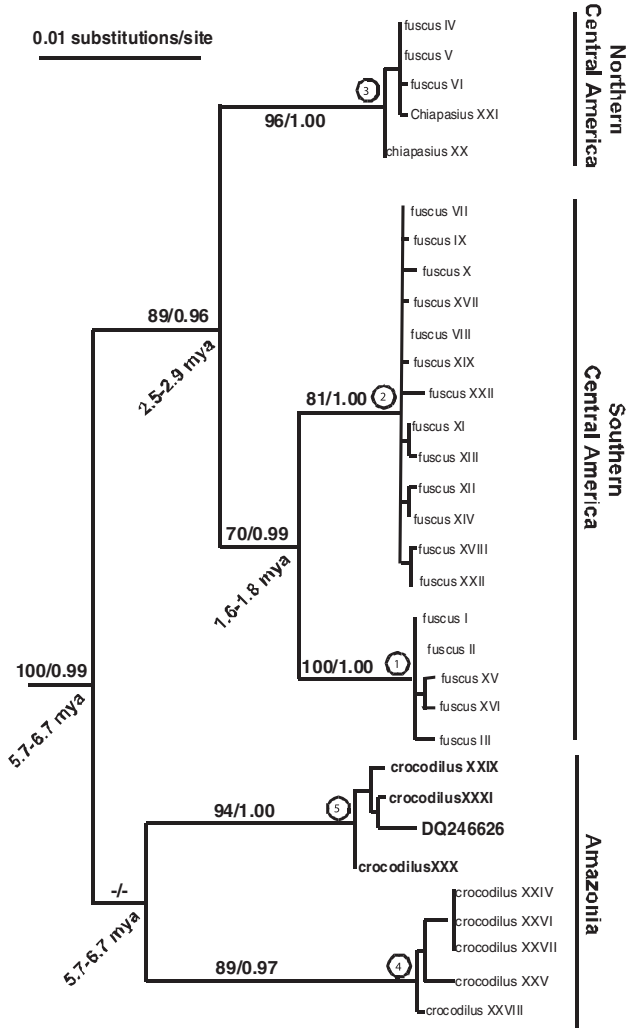

Fig. 2. Maximum likelihood tree inferred for all 31 unique mtDNA haplotypes obtained from *Caiman crocodilus* in this study. Haplotypes consisted of two combined mtDNA sequences: 657 base pairs (bp) of COI and 1,236 bp of Cyt *b*. For each branch on the tree, statistical support is indicated by bootstrap values before the slash and Bayesian marginal posterior probabilities after the slash. Estimated divergence times in millions of years ago (mya) are also indicated for major nodes. Each of the five major clades is numbered arbitrarily as in Figure 1. Clade 5 would contain all Cyt *b* from Vasconcelos et al. (2006) (results not shown), here represented by one Cyt *b* haplotype, GenBank accession number DQ246626. Phylogeny was rooted with one sample each of *Paleosuchus* and *Alligator* (not shown). Cyt *b,* cytochrome *b*.

proportions of variable sites (19.9 and 17.5%, respectively). Relative to *C. crocodilus*, the Cyt *b* sequence from *P. trigonatus* was 31 codons shorter. The Cyt *b* gene from *A. mississippiensis* was two codons shorter, plus it contained a 1-codon gap at nucleotide positions 1167–1169 of the *C. crocodilus* sequence. All sequences could be translated to an apparently functional Cyt *b* protein. Our data showed no signs of saturation when we plotted transitions or transversions against uncorrected genetic distance (results not shown). Therefore, all nucleotide positions were employed in all phylogenetic analyses.

Phylogenetic results

The parsimony-based partition homogeneity test revealed no significant difference in the phylogeny of the Cyt *b* vs. COI gene ($P = 0.899$), as expected for completely linked mitochondrial genes. Visual inspection of NJ trees, ML trees, and Bayesian consensus trees based on either gene sequence alone also suggested no obvious incongruence between the two genes or among methods of phylogenetic inference. For these reasons, all subsequent analyses were based on the combined Cyt *b* and COI data. The best-fit model of evolution for the combined data set was the TVM

+Γ (5-parameter transversional model plus unequal base frequencies and rate variation among sites) (Tamura and Nei, '93). For the combined data partitioned by codon position, the best-fit models were SYM+Γ (symmetrical model) (Zharkikh, '94), HKY (2-parameter model) (Hasegawa et al., '85)+Γ, and GTR (6-parameter model) (Tavaré, '86)+Γ, for first, second, and third positions, respectively.

For the combined data set, our three phylogenetic methodologies produced similar topologies, and the Shimodaira–Hasegawa test showed no significant differences among the NJ, ML, and Bayesian consensus trees ($P > 0.05$). Therefore, in the following discussion we use as our point of reference the phylogenetic tree obtained by ML (Fig. 2).

All mitochondrial DNA sequences from *C. crocodilus* clearly formed a monophyletic group with an average corrected genetic distance of 0.256 separating *Caiman* and *Paleosuchus*. Within *C. crocodilus* we observed five reciprocally monophyletic and well-supported terminal mtDNA clades (numbered 1–5 in Fig. 2) that corresponded to subspecific designations and geography, but with some important exceptions. Two divergent but potentially sister clades were found in Amazonia (northern Peru). These two clades contained only one named subspecies, *C. c. crocodilus*, revealing the presence of a cryptic lineage. The named and the cryptic lineage showed a mean mtDNA divergence of 0.042 (Table 2). Three clades forming a monophyletic group were found in Mesoamerica: one in northern Mesoamerica and two sister clades in southern Mesoamerica (Figs. 1 and 2). Northern vs. southern Mesoamerican clades showed a mean mtDNA divergence of 0.018, whereas within southern Mesoamerica *C. c. fuscus* clade 1 vs. clade 2 showed a mean divergence of 0.011 (Table 2). The lone sample from the Caribbean coast of Colombia (Haplotype XIII) formed a part of the southern Mesoamerican clade (Fig. 1).

The northern Mesoamerican samples consisted of three *C. c. chiapasius* from Mexico and seven *C. c. fuscus* from El Salvador. Despite their current taxonomic status, the mtDNA sequences from these ten individuals formed a clade of five haplotypes with no genetic divergence between samples assigned to different subspecies (Figs. 1 and 2). Furthermore, the northern Mesoamerican clade formed the sister lineage to the *C. c. fuscus* clade from southern Mesoamerica and Caribbean Colombia. Thus, our mtDNA data revealed a lack of concordance between the morphological and molecular assessments of *C. c. fuscus* from northern Mesoamerica. Within the morphological subspecies *C. c. fuscus,* we encountered a pair of reciprocally monophyletic and well-supported clades (Fig. 1). These two clades showed substantial haplotype diversity but no within-clade geographic structure (Table 2).

When we included the published Cyt *b* sequence data from across Amazonia, we find that the entire haplotype network of Vasconcelos et al. (2006) formed a part of clade 5 of *C. c. crocodilus* (Fig. 2). Two haplotypes collected in northern Peru (XXIX and XXXI) were similar to GenBank number DQ246626. This published haplotype represents the most common and widespread haplotype (H1) found by Vasconcelos et al. (2006). Despite their impressive geographic sampling, Vasconcelos et al. (2006) found no evidence of the cryptic lineage labeled ''clade 4'' (Figs. 1 and 2) that we sampled from the Rio Istocohca in central Peru (Table 1).

According to the likelihood ratio test of rate homogeneity, our data failed to reject a molecular clock model of evolution. As crocodylians have a rich and well-studied fossil record, estimating divergence times on our clock-like phylogeny was a straightforward exercise. Assuming the MRCA of *Alligator* and *Caiman* originated 60–70 mya, we obtained the following divergence time intervals. *Paleosuchus* and *Caiman* diverged 35–41 mya. Both the MRCA of *C. crocodilus* and the MRCA of Clades 4 and 5 of Amazonian *C. crocodilus* date back to the Late Miocene 5.7–6.7 mya. The Mesoamerican subspecies, *C. c. chiapasus* and *C. c. fuscus*, last shared a common ancestor in the Pliocene 2.5–2.9 mya. Clades 1 and 2 of *C. c. fuscus* diverged at the dawn of the Pleistocene 1.6–1.9 mya (Fig. 2).

DISCUSSION

In this article we have presented the first species-wide analysis of genetic variation and divergence within *C. crocodilus*. Our data support the biological validity of recognized subspecies while also revealing two additional lineages that were not predicted by subspecific taxonomy, including one South American lineage of late Miocene origin and one Mesoamerican lineage of early Pleistocene origin. Furthermore, our mtDNA data identified one lineage of special concern, *C. c. chiapasius*, which urgently needs more study by biologists interested in preserving units of conservation below the species level.

Conservation genetics

Controversy surrounding the recognition and delimitation of species and subspecies of *Caiman* has significantly impeded conservation and law enforcement efforts aimed at controlling illegal hunting (Thorbjarnarson, '92; Ross, '98). We show here that mtDNA offers an inexpensive and rapid source of objective genetic data that can be combined with morphological and other lines of evidence to clarify confusion surrounding crocodylian taxonomy and species identification. In the case of *C. crocodilus* at least three intraspecific groups that can be identified by both morphology and mtDNA sequence data, making the three lineages obvious candidates for the status of Evolutionarily Significant Units (ESUs) (Moritz, '94). The basal split among the three lineages dates back roughly 6 million years. Identifying ESUs or other intraspecific units is vital to the conservation of *C. crocodilus* because of the intense captive breeding and potential mixing of lineages owing to the commercialization of this species (Thorbjarnarson, '92; Ross, '98; Buitrago, 2001; MacGregor, 2002; Köhler et al., 2006).

The recognition of units of conservation in managed species, such as *C. crocodilus*, allows conservation decision makers to better visualize the species' evolutionary and demographic history, which in turn helps in the elaboration of species management plans (DeSalle and Amato, 2004). For example, mtDNA evidence supporting recognition of *C. c. chiapasius* as a subspecies serves the very practical purpose of drawing the attention of conservation biologists and stakeholders who can best direct limited resources to this distinct evolutionary entity.

In the case of *C. c. chiapasius* our mtDNA results call attention to the urgent need for further investigation and conservation. Not only is this a valid subspecies representing several million years of independent history, the lack of concordance between morphology and genetic data suggests that mtDNA from the *C. c. chiapasius* lineage in Mexico may be introgressing into
C. c. fuscus of El Salvador. The fact that we discovered caimans with *C. c. fuscus* morphology within the geographic range of *C. c. chiapasius* suggests that *C. c. fuscus* may be expanding northward, possibly owing to the high levels of habitat disturbance and degradation in the area. The exact nature of demographic expansion of *C. c. fuscus* and the genetic characterization of the

TABLE 2. Model-corrected pairwise genetic distances among the five clades of Caiman crocodilus inferred in this phylogenetic study, as well as among Caiman, Paleosuchus, and Alligator

Clades	Allmi	CAcr3	CAcrcr4	CAcrcr5	CAcrfuscus1	CAcrfuscus2	Patr
Allmi	0 00 ± 0.00						
CAcr3	0.46253 ± 0.00150	0.00040. ± 0.00034					
CAcrcr4	0.45679 ± 0.00075	0.03848 ± 0.00078	0.00125 ± 0.00121				
CAcrcr5	0.44672 ± 0.00664	0.04551 ± 0.00375	0.04202 ± 0.00245	0.00252 ± 0.00099			
CAcrfuscus1	0.48172 ± 0.00148	0.01845 ± 0.00061	0.03612 ± 0.00079	0.04971 ± 0.00407	0.00071 ± 0.00045		
CAcrfuscus2	0.47586 ± 0.00224	0.01841 ± 0.00064	0.03929 ± 0.00084	0.04168 ± 0.00274	0.01186 ± 0.00070.	0.00109 ± 0.00006	
Patr	0.31547 ± 0.00000	0.24505 ± 0.00108	0.24562 ± 0.00065	0.28942 ± 0.01428	0.25592 ± 0.00154	0.024708 ± 0.00231	0.00000 ± 0.0000

Clade 5 also includes all unique Cyt *b* haplotypes published by Vasconcelos et al. (2006) in their study of Amazonian localities. All other genetic distances are based on combined COI and Cyt *b* sequences

potential hybrid zone between subspecies is not clear and awaits further investigation by microsatellite markers.

Our mtDNA data from South America reveal a cryptic lineage (clade 4) dating back 6 million years, yet whose existence was previously unrecognized. Pending further analyses, this cryptic lineage could merit recognition as a fifth subspecies of *C. crocodilus*. This cryptic lineage likely does not represent a range extension of *C. c. apoporiensis* (from which we were unable to obtain samples) because the latter is the most morphologically distinctive of all subspecies of *C. crocodilus*. Its old age coupled with its apparently quite restricted geographic range may make clade 4 a lineage of special conservation concern.

Phylogeography

We show that the basal divergence of *C. crocodilus* corresponds geographically to the Andean mountains of South America and date this divergence to 5.7–6.7 mya (Figs. 1 and 2). This date agrees well with the timing of the initial development of the northern Andes during the late Miocene (Hoorn et al., 1995; Hooghiemstra et al., 2006). Comparing our results with some additional phylogeographic studies of taxa distributed on both sides of the Andes, we note that the divergence time in *C. crocodilus* matches that of howler monkeys (Cortés-Oritz et al., 2003), whereas in the túngara frog (Weigt et al., 2005), the cane toad (Slade and Moritz, '98), and toucans (Eberhard and Bermingham, 2005) the divergence times seem to correspond more closely with the final upsurge of the northern Andes in the Late Pliocene 2.7 mya (Gregory-Wodzicki, 2000). A third frog, *Leptodactylus fuscus*, likely dispersed from *cis*-Andean South American into Mesoamerican more recently (Camargo et al., 2006).

As an alternative to Andean orogeny, the basal divergence within *C. crocodilus* could be associated with an ancient marine incursion near the present-day Orinoco River (Hoorn, '93; Díaz de Gamero, '96; Hoorn, 2006). This hypothesis would be supported if previously unavailable samples of *C. c. fuscus* from *cis*-Andean Colombia were found to be sister to Mesoamerican *C. c. fuscus* rather than to Amazonian *C. crocodilus*.

Our phylogenetic and divergence time results together suggest that the MRCA of *C. c. fuscus* and *C. c. chiapasius* was already in Mesoamerica by 2.5–2.9 mya. This date corresponds well with the closure of the Pacific–Caribbean seaway by 3 mya (Coates and Obando, '96; Coates et al., 2004), with the Great American Biotic Interchange (Simpson, '40; Marshall et al., '79; Stehli and Webb, '85) and with other phylogeographic studies of certain wide-ranging Neotropical tetrapods (Cortés-Oritz et al., 2003; Eberhard and Bermingham, 2005; Wüster et al., 2005). We find no evidence that *C. crocodilus* entered Mesoamerica before the completion of the Isthmus, as has been suggested for some reptiles, fish, and frogs (Zamudio and Greene, '97; Bermingham and Martin, '98; Perdices et al., 2002; Weigt et al., 2005; Reeves and Bermingham, 2006).

Once in Mesoamerica, *C. crocodilus* appears to have expanded rapidly across the landscape. The basal divergence for the Mesoamerican lineage separates the northernmost *C. c. chiapasius* sample from the rest of the clade. Given that the species originated in South America, this result suggests that Mexico has been occupied by *C. crocodilus* about as long as southern Mesoamerica. Primary freshwater fishes of South American origin show a similar pattern of rapid expansion across Mesoamerica followed by geographic quiescence and a buildup of local or regional population genetic structure (Reeves and Bermingham, 2006).

Significant population genetic structure is seen within *C. c. fuscus*. Clade 2 of *C. c. fuscus* ranges from the Caribbean coast of Colombia, across Panama and into Pacific Costa Rica, yet shows little genetic structure over this wide geographic range (Fig. 2). However, in nearby Caribbean Costa Rica we find the genetically divergent Clade 1 of *C. c. fuscus* that last shared a common ancestor with Clade 2 approximately 1.8 mya. Such a high level of divergence is rather surprising given the lack of any obvious barrier to dispersal along the Caribbean coast between central and western-most Panama (Fig. 1), and given the lack of genetic structure between Pacific Costa Rica and Caribbean Colombia exhibited by Clade 2.

Although we find no obvious geographic barrier separating Clades 1 and 2 of *C. c. fuscus* along the Caribbean coast, numerous other taxa have shown genetic breaks in this same region of Mesoamerica. Crawford et al. (2008) refer to this apparently cryptic geographic barrier as the "Bocas Break." For example, the rain frog, *Craugastor fitzingeri*, shows the same phylogeographic pattern as *C. c. fuscus* in which Caribbean Costa Rica is distinct from a unified Pacific Costa Rica + Central Panama. The time of divergence separating Caribbean and Pacific populations of

C. c. fuscus in Costa Rica corresponds to the estimated age of the intervening mountains (the Tilarán range of northern Costa Rica) at 1–2 mya (Denyer et al., 2000). Thus, *C. c. fuscus* in Caribbean Costa Rica could have been derived from Pacific Costa Rica rather than Caribbean Panama, as was found for the pygmy rain frog (Wang et al., 2008).

In conclusion, our results revealed that the genus *Caiman* is far more diverse than previously thought, especially in Central America where we found significant population structure. In many cases, there were no obvious geographic barriers between distinct mtDNA clades in *C. crocodilus*, suggesting the possibility of environmental barriers in promoting or maintaining distinct genetic entities. We suspect that the introgression of *C. c. chiapasius* into *C. c. fuscus* may be owing to anthropogenic activities that destroyed habitat and promoted contact among caiman populations that had been separated for millions of years. Now that we have a better understanding of the number and distribution of major clades within *C. crocodilus* from mtDNA data, we advocate that other genetic markers (e.g., like microsatellites or AFLPs) are needed to better assess the interactions and potential hybridization that appears to be occurring among these major units, along with more intense geographical sampling among *cis-* and *trans-* Andean populations and the Apoporis River population. We strongly advocate the design and implementation of a coherent management plan that is based on recognized conservation units.

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