

# A TIME-CALIBRATED SPECIES TREE OF CROCODYLIA REVEALS A RECENT RADIATION OF THE TRUE CROCODILES

Jamie R. Oaks<sup>1,2</sup>

<sup>1</sup>Biodiversity Institute and Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, Kansas 66045

<sup>2</sup>E-mail: joaks1@ku.edu

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True crocodiles (*Crocodylus*) are the most broadly distributed, ecologically diverse, and species-rich crocodylian genus, comprising about half of extant crocodylian diversity and exhibiting a circumtropical distribution. *Crocodylus* traditionally has been viewed as an ancient group of morphologically conserved species that originated in Africa prior to continental breakup. In this study, these long-held notions about the temporal and geographic origin of *Crocodylus* are tested using DNA sequence data of 10 loci from 76 individuals representing all 23 crocodylian species. I infer a time-calibrated species tree of all Crocodylia and estimate the spatial pattern of diversification within *Crocodylus*. For the first time, a fully resolved phylogenetic estimate of all Crocodylia is well-supported. The results overturn traditional views of the evolution of *Crocodylus* by demonstrating that the true crocodiles are not “living-fossils” that originated in Africa. Rather, *Crocodylus* originated from an ancestor in the tropics of the Late Miocene Indo-Pacific, and rapidly radiated and dispersed around the globe during a period marked by mass extinctions of fellow crocodylians. The findings also reveal more diversity within the genus than is recognized by current taxonomy.

**KEY WORDS:** Biogeography, *Crocodylus*, Dirichlet process, multispecies coalescent, relaxed clock.

Modern crocodylian systematics has been dominated by investigations of higher level relationships aimed at resolving the disparity between morphological and molecular data, especially regarding the phylogenetic placement of the true gharial (*Gavialis gangeticus*) (e.g., Densmore and Dessauer 1984; Norell 1989; Gatesy and Amato 1992; Aggarwal et al. 1994; Poe 1996; Brochu 1997; Gatesy et al. 2003; Harshman et al. 2003; Janke et al. 2005; Willis et al. 2007; Piras et al. 2010). Consequently, no study has provided full resolution of the interspecific relationships within the most broadly distributed, ecologically diverse, and species-rich crocodylian genus, *Crocodylus*. Most molecular phylogenetic studies of Crocodylia included only a subset of the 11 named species of *Crocodylus* (Gatesy and Amato 1992; Hass et al. 1992; Gatesy et al. 1993; Aggarwal et al. 1994; Brochu 1997; Brochu and Densmore 2000; Gatesy et al. 2003; Harshman et al. 2003; Schmitz et al. 2003; Janke et al. 2005; McAliley

et al. 2006; Li et al. 2007; Willis et al. 2007; Feng et al. 2010; Meganathan et al. 2010; Man et al. 2011). Most studies that included all 11 species were unable to resolve interspecific relationships and yielded largely incongruent results (Densmore 1983; Densmore and Owen 1989; Densmore and White 1991; White 1992; White and Densmore 2000; Poe 1996; Gratten 2003; Gatesy et al. 2004; Gatesy and Amato, 2008). The only fully resolved phylogenetic estimate of *Crocodylus* is based solely on mitochondrial DNA (Meredith et al. 2011).

## LIVING FOSSILS FROM AFRICA?

Traditionally, *Crocodylus* has been stereotyped as an ancient group of morphologically conserved species (i.e., “living fossils”) that originated in Africa during the Cretaceous (Lydekker 1886; Mook 1927, 1933; Kälin 1955; Sill 1968); their current circumtropical distribution has been explained by continental drift

(Brooks 1979; Brooks and O'Grady 1989). Early molecular studies demonstrated relatively low levels of variation among species of *Crocodylus*, thereby suggesting that the genus might be younger than traditionally thought (Densmore 1983; Densmore and White 1991; White 1992). Subsequent reassessment of paleontological data with rigorous cladistic methods (Brochu 1997, 2000b; Salisbury and Willis 1996) demonstrated that paleontologists, basing their taxonomy on general gestalt and plesiomorphic characters, had applied the name "*Crocodylus*" to a wide variety of distantly related species (Brochu 2000a, b). Fossils unequivocally identifiable as *Crocodylus* do not appear until the late Miocene or later (Mook 1933; Molnar 1979; Miller 1980; Lydekker 1886; Willis 1997; Brochu 2000a; Mead et al. 2006; Salisbury et al. 2006; Delfino et al. 2007).

Estimates of the age of *Crocodylus* based on molecular divergence dating are limited to analyses of mitochondrial data that included only two samples from the genus (*C. niloticus* and *C. porosus*) and yielded highly variable results (Janke et al. 2005; Roos et al. 2007). Janke et al. (2005) estimated the time of divergence between *C. niloticus* and *C. porosus* to be 39–9 million years ago (mya), extending into the Eocene, and the divergence between Gavialinae and Crocodylinae (Table S1) to be 101–68 mya; these results suggest that much of the diversification within the genus may have occurred at a time when the continents were much less dispersed. In contrast to these results, Roos et al. (2007) estimated the same divergences to be approximately 19–9 mya and 60–34 mya, respectively, depending on the methods used. Given the accumulating evidence against the traditional hypothesis of an ancient *Crocodylus*, and paucity of sequence-based dating analyses, there is a clear need for a robust, time-calibrated phylogenetic estimate of Crocodylia to provide a better understanding of the temporal framework of the diversification of *Crocodylus*.

Although the ancientness of *Crocodylus* has been questioned, Africa is still thought to be the geographic center of origin (Brochu 2000a, b, 2007; Delfino et al. 2007). The "out-of-Africa" paradigm stems from the basal phylogenetic relationships within Crocodylinae supported by morphological data (Brochu 2000a; 2007) and some molecular data (Densmore 1983; Densmore and White 1991; Poe 1996; Gatesy et al. 2004; McAliley et al. 2006; Li et al. 2007) that place two African taxa (*Osteolaemus* and *Mecistops*) as consecutive outgroups to *Crocodylus*. However, this topology is likely erroneous; other molecular data strongly support *Mecistops* as sister to *Osteolaemus* (White 1992; White and Densmore 2000; Schmitz et al. 2003; Gatesy et al. 2004; McAliley et al. 2006; Li et al. 2007; Willis et al. 2007; Gatesy and Amato 2008; Feng et al. 2010; Man et al. 2011). Thus, these two genera might represent a single, relictual sister group to *Crocodylus*. Furthermore, most fossils referred to as *Crocodylus* from the Miocene of Africa are either

*Mecistops cataphractus* (Tchernov 1986; Brochu 2000a) or have been phylogenetically placed as extinct species outside the genus by cladistic estimates (Brochu 2007; Brochu et al. 2010). Unequivocal fossils of *Crocodylus* in Africa are younger than the oldest fossils of this genus in Asia (Lydekker 1886; Mook 1933; Brochu 2000a), Australia (Molnar 1979; Willis 1997), and the New World (Miller 1980). Thus, depending on the phylogenetic placement of *M. cataphractus*, there may be little basis for the "out-of-Africa" paradigm.

In this study, I collect a large, multilocus sequence dataset representing all extant crocodylian species, and test traditional ideas concerning the ancient and African origin of *Crocodylus*. I infer a time-calibrated phylogeny using both traditional and coalescent-based phylogenetic models to resolve the relationships within Crocodylinae and obtain a robust estimate of the age of *Crocodylus*. I also infer the biogeographical history of the genus under a variety of evolutionary models and test the "out-of-Africa" hypothesis.

## Methods

Full details of data collection and methodology are contained in Supporting information. I obtained tissue samples from one to ten individuals for all 23 described crocodylian species (Table S2), and collected DNA sequences from four regions of the mitochondrial genome and nine nuclear loci. After assessing congruence among the loci, I concatenated the data into an alignment of 7282 sites for 76 individuals (*Dryad* doi: 10.5061/dryad.5k9s0; GenBank accession nos. JF314862–315859).

## PHYLOGENETIC ANALYSES

To estimate how substitution rates vary across the alignment, I analyzed the data under a Bayesian Dirichlet process prior model (DPP; source code provided by J. Huelsenbeck; Huelsenbeck and Suchard 2007). I calculated which sampled partition from the posterior of the DPP analysis minimized the distance to all other partitions in the posterior sample; I used this partition, with the best-fit model of nucleotide substitution applied to each subset, in all subsequent phylogenetic analyses.

Using the rich crocodylian fossil record to inform node age constraints, I estimated a rooted, ultrametric, time-calibrated phylogeny with BEAST (version 1.5.4; Drummond and Rambaut 2007), applying separate uncorrelated lognormal relaxed-clocks to each subset. The Alligatorinae–Caimaninae split between 71 and 64 mya is considered among the best vertebrate fossil-calibration points (Muller and Reisz 2005); thus, a normally distributed prior was used on this node that placed 95% of the probability density within this range. Additionally, a conservative upper bound of 90 mya was placed on the root of Crocodylia to extend the likely Campanian origin of Crocodylia

(Brochu 2003; Salisbury et al. 2006) by 6.5 my. Separate analyses also were done with the upper bound extended to 100 mya to examine the effect of this prior.

To relax the assumption of congruence among the gene trees of each locus, I used the multispecies coalescent model of \*BEAST (BEAST version 1.5.4; Heled and Drummond 2010) to estimate the species tree of Crocodylia. The same model of nucleotide substitution as the standard BEAST analysis was applied, including the partitioning of the alignment into relaxed clock subsets. However, the gene trees were estimated independently (conditional on the species tree) for each of the 10 loci. Also, the same age constraints were placed on nodes of the species tree. I used GARLI (version 0.96; Zwickl 2006) to infer the maximum-likelihood (ML) phylogeny and assessed support via bootstrapping.

I estimated the probability of monophyly of (1) *Crocodylus* + *Mecistops*, (2) the Australasian species, (3) *C. niloticus*, (4) *C. siamensis* + *C. porosus*, (5) *C. novaeguineae*, as well as alternate rootings of Crocodylia, by the proportion of trees in the posterior sample of the BEAST and \*BEAST analyses that were congruent with each hypothesis. I also tested these phylogenetic hypotheses with an approximately unbiased (AU) test (CONSEL; Shimodaira and Hasegawa 2001) using site-wise likelihoods optimized on sets of unique topologies obtained from unconstrained and constrained ML searches (using RAXML; Stamatakis 2006). See Supporting information for full details.

### BIOGEOGRAPHIC ANALYSES OF CROCODYLUS

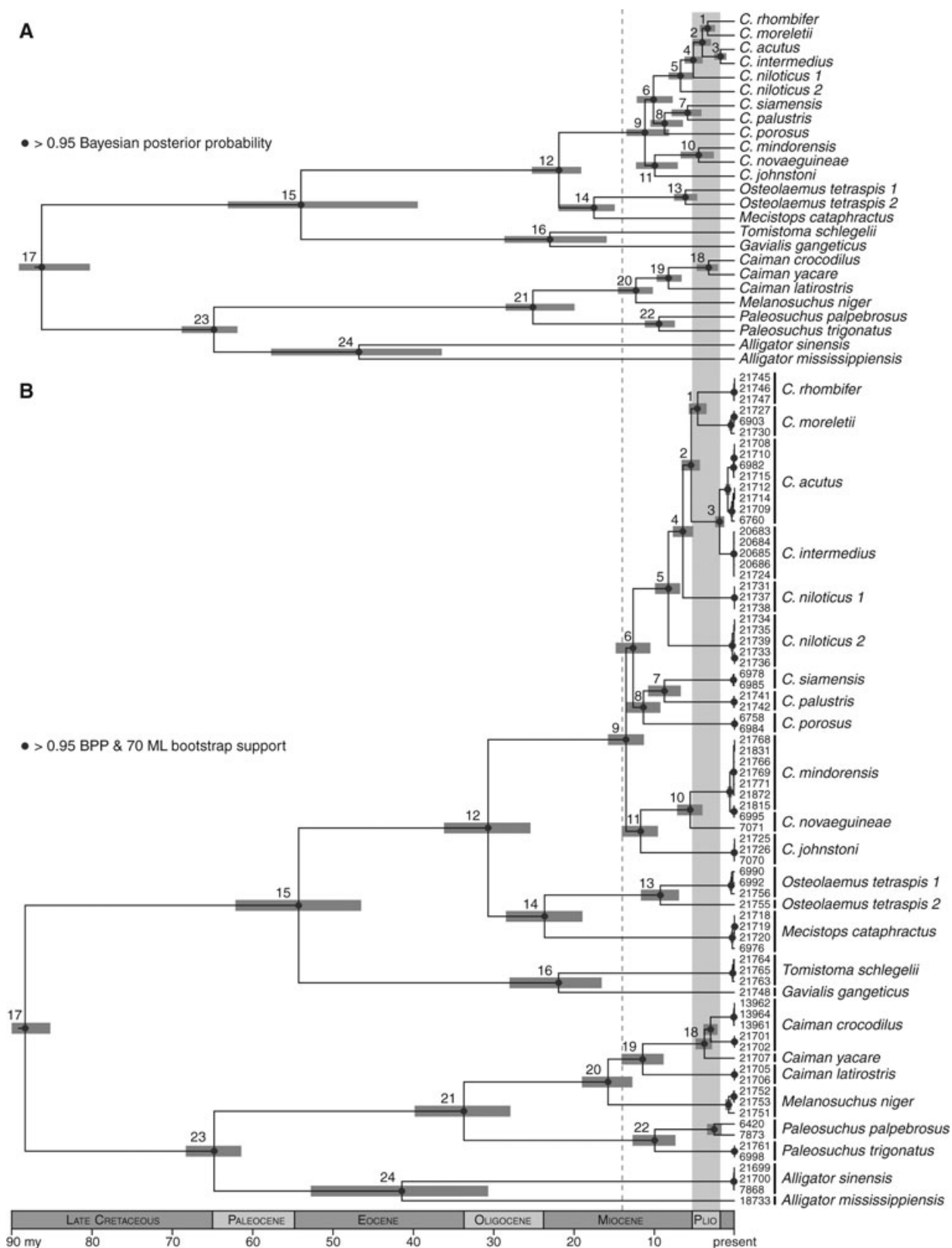
Biogeographic ancestral-area reconstructions were performed on *Crocodylus*, with *Osteolaemus* and *Mecistops* as outgroups. The distribution of each crocodyline species was coded as a character with three states (Neotropical, African, and Australasian) based on their distribution (Fig. S1). After conducting a model-selection procedure to determine the best model of character evolution (detailed in Supporting information), I estimated ML ancestral area reconstructions in BayesTraits (Pagel 1999) under the selected model using the majority-rule consensus topology with mean node ages from the \*BEAST posterior of sampled species trees. I also estimated ML reconstructions on this consensus tree under the dispersal-extinction-cladogenesis (DEC) model in LaGrange (version 2.0.1; Ree and Smith 2008). To assess support for the out-of-Africa hypothesis, I reran all ML analyses with the basal-most node of *Crocodylus* constrained to each of the three character states, and compared the fit of these models to the respective unconstrained analysis using the change in likelihood score. I estimated Bayesian ancestral-area reconstructions with BayesTraits (Pagel et al. 2004) under the same best-fit model selected for the ML analyses, and a reversible-jump model that integrates over models of character evolution, thus accommodating uncertainty in model selection. Rather than using the consensus tree, I used

the posterior of sampled species trees from the \*BEAST analysis in all Bayesian reconstructions. Three additional Bayesian analyses were run under each model with the basal-most node of *Crocodylus* constrained to each of the three possible character states. I assessed the strength of support against each of these constrained basal states by calculating the Bayes factor (approximated using harmonic mean likelihood scores) in comparison with the unconstrained model.

## Results

For the first time, fully resolved relationships within *Crocodylus* are well-supported; all concatenated phylogenetic analyses in BEAST, GARLI, and the DPP program yielded the same interspecific relationships with strong support (Fig. 1B and Table 1). Among the strongly supported relationships are the following: (1) *M. cataphractus* is sister to *Osteolaemus*; (2) the Australasian species are paraphyletic; (3) *Crocodylus niloticus* is split into two distinct, nonsister species; (4) one of the *C. novaeguineae* falls out with *C. mindorensis*; and (5) *C. siamensis* is sister to *C. palustris*. The monophyly of *C. niloticus*, *C. novaeguineae*, *Mecistops* + *Crocodylus*, and *C. siamensis* + *C. porosus* is rejected by an AU test ( $P = 0.002$ ,  $P = 6 \times 10^{-48}$ ,  $P = 0.002$ , and  $P = 0.026$ , respectively). Likewise, no posterior tree samples from the BEAST analyses are consistent with any of these constraints. The AU test is unable to reject the monophyly of the Australasian species ( $P = 0.393$ ), but the posterior probability of this relationship is only  $9.53 \times 10^{-4}$  and  $6.50 \times 10^{-4}$  for the BEAST analysis with a maximum age constraint of 90 my and 100 my, respectively.

Based on these concatenated results, I assigned most individuals to their named species when setting up the \*BEAST species tree analyses. Among the exceptions are the following: (1) The two clades of *C. niloticus* are considered separate species; (2) the *C. novaeguineae* (LSUMZ H-6995) that fell out with *C. mindorensis* is considered to be the latter species; and (3) one *Osteolaemus tetraspis* (LSUMZ H-21755) is considered to be a separate species from its conspecifics, given the deep divergence between them (Fig. 1B). The results of the \*BEAST species tree analysis strongly support the same interspecific relationships as the concatenated analyses (Fig. 1 and Table 1), including the same relationships within *Crocodylus*. None of the posterior species tree samples from the \*BEAST analyses is consistent with the monophyly of *C. niloticus*, *Mecistops* + *Crocodylus*, or *C. siamensis* + *C. porosus*. The estimated posterior probability of monophyly of the Australasian species is  $2.75 \times 10^{-3}$  and zero for the \*BEAST analysis with a maximum age constraint of 90 my and 100 my, respectively. The BEAST and \*BEAST analyses unambiguously infer the root of Crocodylia at the branch between Crocodyliidae and Alligatoridae (Fig. 1); none of the trees in the posterior



**Figure 1.** The majority-rule consensus tree with mean node ages from the (A) \*BEAST posterior sample of species trees and (B) BEAST posterior sample. Bars at nodes represent the 95% highest posterior density of the node age. The numbers at internal nodes correspond to Table 1. The numbers at terminal nodes refer to LSUMZ ID numbers in Table S2. The Tertiary, after the mid-Miocene climatic optimum (dashed line), was marked by extinctions of crocodilians, which culminated in the Pliocene (gray bar) (Markwick 1998).

**Table 1.** Node statistics. Node numbers correspond to Figure 1. Node age posterior means and 95% highest posterior densities (HPD) are from the \*BEAST and BEAST analyses with 90 and 100 my upper limits on the root age. Posterior probabilities are from the Dirichlet process prior (DPP) model analysis, and the \*BEAST and BEAST analyses with a 90 my upper limit on root age (the values from the analyses with the 100 my limit were nearly identical). The bootstrap (BS) percentage for each node from the Garli analysis is also given.

Node	Node ages											
	Species tree				Concatenation				Node support			
	90 my max		100 my max		90 my max		100 my max		Posterior probability			
	Mean	95% HPD	Mean	95% HPD	Mean	95% HPD	Mean	95% HPD	*BEAST	BEAST	DPM	BS
1	3.42	2.49–4.37	3.8	2.47–5.17	4.62	3.51–5.70	5.01	3.84–6.23	0.95	1	0.94	75
2	4.08	2.98–5.31	4.61	3.37–6.56	5.41	4.34–6.61	5.87	4.73–7.17	1	1	1	93
3	1.79	1.06–2.57	1.89	1.10–2.72	1.87	1.30–2.44	2.01	1.45–2.61	1	1	1	100
4	5.21	4.05–6.31	5.73	4.37–7.35	6.46	5.17–7.70	6.97	5.55–8.27	1	1	1	100
5	6.82	5.28–8.32	7.35	5.69–9.41	8.26	6.80–9.92	8.93	7.33–10.69	1	1	1	100
6	10.21	7.80–12.32	10.78	8.66–12.81	12.65	10.50–14.82	13.74	11.53–16.10	1	1	1	81
7	5.95	4.19–7.94	6.82	4.54–8.91	8.75	6.72–10.78	9.53	7.49–11.86	1	1	1	100
8	8.82	6.52–10.60	9.6	7.33–11.65	11.37	9.26–13.45	12.36	10.17–14.66	1	1	1	90
9	11.3	8.28–13.61	11.69	9.47–13.72	13.53	11.31–15.78	14.68	12.35–17.21	1	1	1	100
10	4.53	2.63–6.81	4.67	3.37–6.01	5.54	4.02–7.18	5.98	4.47–7.64	1	1	1	100
11	10.06	7.17–12.41	10.31	7.93–12.45	11.71	9.57–14.06	12.7	10.42–15.27	1	1	1	100
12	22.1	19.31–25.51	26.19	22.46–31.71	30.7	25.43–36.19	33.78	27.96–39.85	1	1	1	100
13	6.21	4.72–7.64	7.6	5.77–8.97	9.26	6.93–11.67	10.07	7.43–12.54	1	1	1	100
14	17.71	15.08–22.15	21.88	16.94–27.68	23.67	18.95–28.48	25.98	21.04–31.67	1	1	1	100
15	54.53	39.84–63.73	62.23	56.30–69.85	54.28	46.50–62.15	60.78	50.68–69.91	1	1	1	100
16	23.23	16.10–28.97	25.71	19.24–29.04	21.93	16.56–28.03	24.06	18.21–30.52	1	1	1	100
17	87.14	81.08–90.00	98.33	95.41–100.00	88.32	85.19–90.00	97.58	93.25–100.00	1	1	NA	NA
18	3.28	2.09–4.79	3.05	2.23–4.02	3.79	2.84–4.87	4.04	3.01–5.08	1	1	1	100
19	8.34	6.69–9.81	8.67	7.06–10.89	11.46	8.86–14.08	12.21	9.48–14.81	1	1	1	100
20	12.43	10.31–14.68	13.1	10.60–16.87	15.77	12.75–19.02	16.76	13.45–20.06	1	1	1	100
21	25.37	20.14–28.81	29.01	24.85–32.55	33.71	27.93–39.84	35.51	29.96–41.48	1	1	1	100
22	9.53	7.54–11.31	9.57	7.55–11.99	9.96	7.39–12.72	10.75	8.14–13.64	1	1	1	100
23	65.48	62.53–69.53	64.65	61.76–67.23	64.79	61.43–68.33	65.39	62.04–68.76	1	1	1	100
24	47.25	36.83–58.28	39.88	35.29–43.86	41.44	30.70–52.76	42.4	32.14–52.82	1	1	1	98

sample from these analyses place the root elsewhere. My node age estimates were affected little by choice of the upper age constraint on the root of Crocodylia (Table 1).

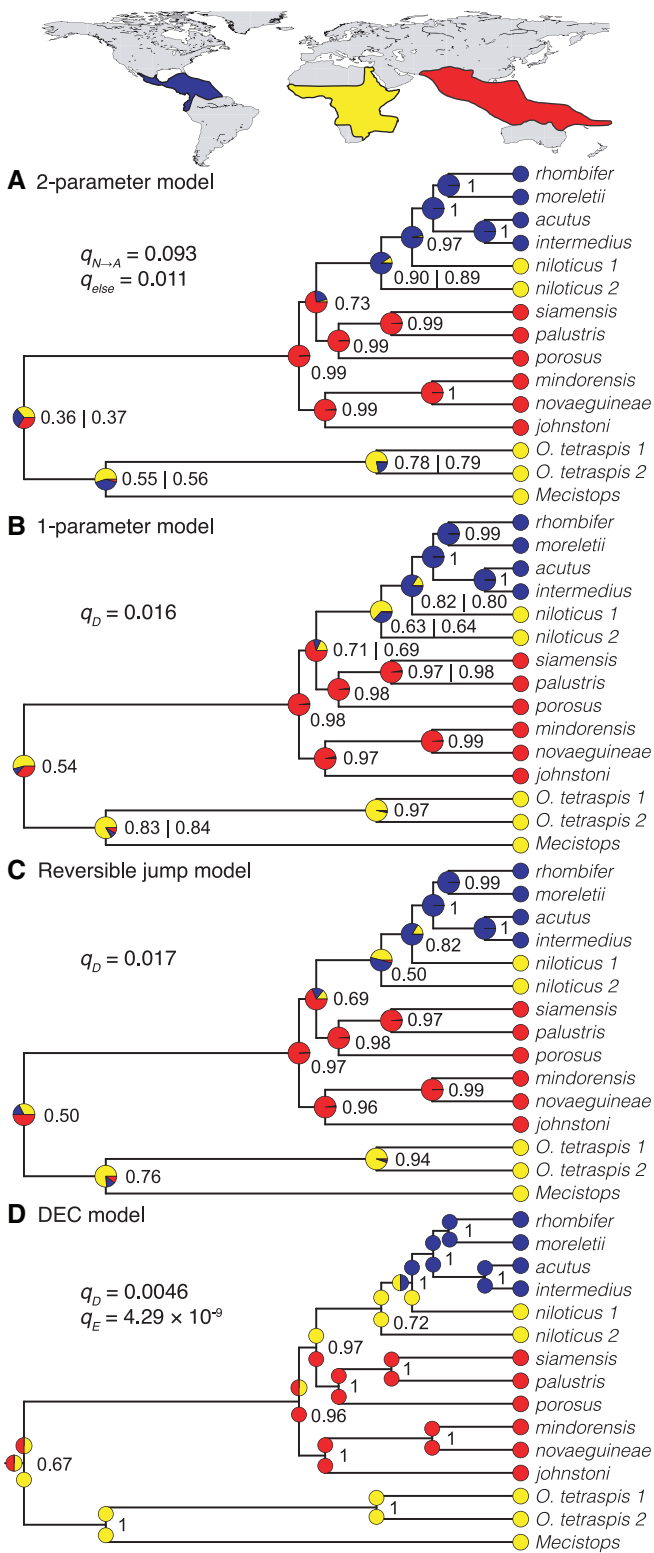
### BIOGEOGRAPHY OF *CROCODYLUS*

My results support Australasia as the center of origin of *Crocodylus*, and reject the “out-of-Africa” hypothesis (Fig. 2 and Table 2). Ambiguity is associated with selecting the best-fit model of character evolution for ancestral-area reconstructions in BayesTraits (Table S3). The Akaike information criterion (AIC; Akaike 1974) selected a model with two transition rate parameters—Neotropics to Africa ( $q_{N \rightarrow A}$ ) and all other transitions ( $q_{else}$ )—whereas the likelihood ratio test (LRT) narrowly failed to reject the simpler one-parameter model ( $P = 0.08$ ; Table S3). Because of this dis-

crepancy between the model selection criteria, I conducted ML and Bayesian analyses in BayesTraits with both models; the results are shown in Figure 2, along with the reconstructions under reversible-jump and DEC models.

All ancestral-area reconstructions under the one-parameter, two-parameter, and reversible-jump models strongly support the most recent common ancestor (MRCA) of *Crocodylus* originating in Australasia (Figs. 2A–C). The reconstruction under the DEC model supports a vicariant range inheritance scenario in which the ancestor of *Crocodylus* was distributed across Australasia and Africa. When the basal node of *Crocodylus* is constrained to each of the possible character states, all four models reject the hypotheses in which the MRCA originated in either Africa or the Neotropics (Table 2).





**Figure 2.** Results of the biogeographic character-state reconstructions under the (A) two-parameter, (B) one-parameter, and (C) reversible jump models in BayesTraits, and (D) DEC model in Lagrange. For (A) and (B), the pie graphs represent the maximum likelihood (ML) relative probabilities of each state at each node, and the value for the most probable state are given in the

**Table 2.** Results of biogeographic constraints of the MRCA of *Crocodylus*. The changes in likelihood score ( $\Delta\ln L$ ) and Bayes factors (BF) are relative to the unconstrained analysis. Results for the two-parameter (2-P), one-parameter (1-P), reversible-jump (RJ), and dispersal-extinction-cladogenesis (DEC) models are given. I consider  $\Delta\ln L > 2$  (Edwards 1972) and  $BF > 6$  (Kass and Raftery 1995) as strong support against the hypothesis.

Constraint	2-P model		1-P model		RJ model	DEC
	$\Delta\ln L$	BF	$\Delta\ln L$	BF	BF	$\Delta\ln L$
Africa	4.52	9.10	3.65	6.94	8.73	10.98
Neotropics	4.59	9.45	3.65	7.86	4.82	13.83
Australasia	0.01	0.37	0.03	0.14	0.59	3.90

Discussion

This is the largest molecular dataset used to infer the phylogeny of Crocodylia to date, both in terms of loci and taxon sampling. The result is a fully resolved, strongly supported, time-calibrated species tree of all crocodylian species (Fig. 1A and Table 1).

RELATIONSHIPS WITHIN CROCODYLINEAE

My phylogenetic results strongly support the following interesting relationships within Crocodylinae (Fig. 1 and Table 1): (1) monophyly of *Mecistops* and *Osteolaemus*, (2) paraphyly of the Australasian species, (3) paraphyly of multiple species within *C. niloticus*, (4) paraphyly of *C. mindorensis* samples, and (5) relationships among the species from mainland Asia that are different from recent results based on mitochondrial data.

Placement of mecistops

*Mecistops* is sister to *Osteolaemus*, and the pair represents a single, divergent sister group to the true crocodiles (Fig. 1 and

following format: ML probability | Bayesian mean posterior probability (only one value is given at nodes where these values are equal). For (C), the pie graphs represent the posterior mean relative probabilities of each state, and the value for the most probable state is shown. For (D), the most probable range inheritance scenarios are shown at each node along with their probabilities. The ancestral range is depicted as a circle to the left of the node only if the state changes along the branch. The circles above and below each node represent the range inherited by each lineage. Bicolored circles represent broad ranges encompassing both geographic states. ML estimates of the rate parameters (transitions/my) are given for A, B, & D, and Bayesian median estimate for C; they are denoted as follows: (A)  $q_{N \rightarrow A}$  is the rate of transition from Neotropics to Africa and  $q_{else}$  is the rate of all other transitions, (B, C, & D)  $q_D$  is the rate of dispersal, and  $q_E$  is the rate of extinction.

Table 1). This finding corroborates previous estimates based on other molecular data (White 1992; White and Densmore 2000; Schmitz et al. 2003; Gatesy et al. 2004; McAliley et al. 2006; Li et al. 2007; Willis et al. 2007; Gatesy and Amato 2008; Feng et al. 2010; Man et al. 2011) and refutes the sister relationship between *Mecistops* and *Crocodylus* supported by morphological data (Brochu 2000a, b; 2007; Brochu et al. 2010) and other molecular data (Densmore 1983; Densmore and White 1991; Poe 1996; Gatesy et al. 2004; McAliley et al. 2006; Li et al. 2007). Finding a sister relationship between *Mecistops* and *Osteolaemus* is not surprising, but verifying these African genera represent a single divergent sister group (rather than consecutive sister taxa) to *Crocodylus* is important to estimating the geographic origin of the genus.

#### Paraphyly of Australasian species

In my results, the African + Neotropical clade of *Crocodylus* is nested within the Australasian species (Fig. 1 and Table 1), providing support for an Australasian origin of *Crocodylus*. This relationship is in contrast to estimates based on morphological (Brochu 2007; Brochu et al. 2010) and mitochondrial (Man et al. 2011; Meredith et al. 2011) data, which support a basal split between the Australasian and African + Neotropical clades. Given the short branches at the base of *Crocodylus* (Fig. 1), the strongly supported conflict between my results and those based on whole mitochondrial genomes (Man et al. 2011; Meredith et al. 2011) may likely be due to incomplete lineage sorting between the earliest speciation events within the genus. These results emphasize the importance of sampling multiple loci when the species tree is the parameter of interest. By accurately resolving the basal relationships among *Crocodylus*, my results are integral to understanding the biogeographic history of the genus.

#### Multiple species of African Nile crocodile

My results clearly show that *Crocodylus niloticus* comprises two distinct species. These species are not sisters, but rather represent consecutive outgroups to the New World clade (Fig. 1). Meredith et al. (2011) also found strong support for this relationship based on whole mitochondrial genomes, but stressed that their results might only reflect the mitochondrial gene tree. My results, based on 10 loci, corroborate the paraphyly of at least two distinct species within *C. niloticus*. This also is congruent with recent studies based on microsatellite data that showed substantial differentiation among *C. niloticus* from different regions of Africa (Hekkala et al. 2010).

#### Paraphyly of the Philippine crocodile

The New Guinea freshwater crocodile, *C. novaeguineae*, is nested within the Philippine freshwater crocodile, *C. mindorensis*. However, one of the two individuals of *C. novaeguineae* (LSUMZ

H-7071) is quite divergent (6.8–2.6 mya) from the clade comprised of the other individual (LSUMZ H-6995) and *C. mindorensis* (Fig. 1B), suggesting there are two species represented on these islands (Fig. 1 and Table 1). Given that all the samples of *C. mindorensis* were collected from the Philippines (Table S2), my results suggest that there are populations of *C. mindorensis* in New Guinea. However, both samples of *C. novaeguineae* used in this study are from captive animals, so the results should be treated with caution.

#### Relationships among species from mainland Asia

Meganathan et al. (2010) recently concluded, based on whole mitochondrial genomes and one nuclear locus from one individual per species, that *C. porosus* and *C. siamensis* are sister species; their results were ambiguous concerning the placement of *C. palustris*. The results of Meredith et al. (2011) also placed one of the sampled *C. siamensis* within *C. porosus*, whereas the other sample was sister to *C. palustris*. My findings, based on 10 loci and multiple individuals per species, clearly show that *C. siamensis* is sister to *C. palustris*, and that *C. porosus* is sister to both species (Fig. 1 and Table 1). As suggested by Meredith et al. (2011), the relationship between *C. siamensis* and *C. porosus* may be an artifact of using samples from captive *C. siamensis* × *C. porosus* hybrids; these species are commonly interbred in captivity (Fitzsimmons et al. 2002). Additional samples from wild populations of the Australasian species of *Crocodylus* are needed to better understand the diversity and interspecific relationships in this region.

#### TEMPORAL AND SPATIAL HISTORY OF CROCODYLUS

The inferred age of the most recent common ancestor of *Crocodylus* is far too recent (approximately 13.6–8.3 mya; Table 1) for vicariance via continental drift to explain the circumtropical distribution of the genus. This study is not the first to suggest a relatively young and dispersal-mediated history of *Crocodylus*; the recent realization that the genus is absent from the fossil record until the Late Miocene and has relatively low levels of genetic variation has led to this hypothesis (Densmore 1983; White 1992; Brochu 2000a; 2000b). However, this is the first study to estimate robustly the timing of diversification of the entire genus, and solidly reject hypotheses of vicariance; also, it is one of the first estimates of a fossil-calibrated species tree of any group.

The recent age of the MRCA mandates a dynamic, dispersal-mediated colonization of the tropics by the crocodiles. With the exception of the unconstrained DEC model (discussed in detail below), all of the spatial reconstruction analyses strongly support Australasia as the center of origin of *Crocodylus* (Fig. 2 and Table 2), rejecting the long-held “out-of-Africa” paradigm of *Crocodylus* biogeography. Beyond the Australasian origin, the spatial pattern of the colonization of the rest of the tropics is not

entirely clear. Depending on the model used for reconstruction, there is support for two general biogeographic patterns: (1) The two-parameter model supports dispersal from Australasia to the Neotropics, followed by dispersal to Africa; and (2) the one-parameter and DEC model support dispersal from Australasia into Africa, followed by dispersal to the Neotropics. The estimate under the reversible-jump model is a compromise between these two scenarios.

### *The Australasia to Neotropics scenario*

The two-parameter model supports trans-Pacific dispersal from Australasia to the New World 12.3–7.8 mya, followed by two invasions of Africa from the Neotropics, between 8.3 and 4.1 mya (Fig. 2A). A trans-Pacific colonization of the New World from the Indo-Pacific never has been considered for *Crocodylus*, however, there are several lines of evidence that suggest it might be possible. The first is the fossil record of *Crocodylus*; the Australasia→Neotropics→Africa biogeographic scenario is congruent with the first appearance of fossil *Crocodylus* within these regions. The oldest *Crocodylus* fossils are of *C. palaeindicus* from the Late Miocene of the Indian subcontinent and Southeast Asia (Brochu 2000b). The next oldest *Crocodylus* fossils are those of *C. porosus* from Australia (4.5–4 mya) (Molnar 1979; Willis 1997), which is approximately the same time that *Crocodylus* appears in the fossil record of the Neotropics (~4 mya) (Miller 1980). Last, *Crocodylus* do not appear in the fossil record of Africa until 3–2 mya (Tchernov 1986). This congruence might be an artifact of sampling error and/or bias in the fossil record, but warrants consideration given that crocodiles are relatively common in the fossil record.

The second line of evidence is from the distribution of the estuarine crocodile, *C. porosus*, which extends well into the Pacific, to the Islands of Solomon, Palau, Vanuatu, and historically Fiji (Groombridge 1987; Neill 1971; Pope 1955; Ross 1998). Additionally, the extinct crocodylian lineage Mekosuchinae was widespread in the Pacific up until the Pleistocene (Mead et al. 2002; Molnar et al. 2002), further demonstrating the oceanic islands of the Pacific contained suitable crocodylian habitat. Furthermore, *C. porosus* frequently is observed at sea and has been documented as far as 800 km (Bustard and Choudhury 1982) and 1360 km (Allen 1974) from land. Thus, it is possible to imagine a rare crossing of the Pacific Ocean by a highly vagile and marine-adapted ancestor similar to the extant estuarine crocodile.

A third line of evidence comes from the marine molecular phylogenetic literature; several taxa show genetic patterns consistent with west-to-east dispersal across the Pacific (e.g., gastropods, sea urchins, and wrasse fish; Palumbi 1997; Lessios et al. 1998, 1996; Lessios et al. 1999; Barber and Bellwood 2005; Latiolais et al. 2006) and Atlantic (e.g., mangroves and sea urchins; Lessios et al. 1999; Nettel and Dodd 2007). Most ap-

plicable to crocodylians is the example from mangroves (Nettel and Dodd 2007), which provide habitat for several *Crocodylus* species. Despite the stark life-history differences between these estuarine/marine taxa and crocodiles, they demonstrate that an Australasia→Neotropics→Africa route of dispersal is not unprecedented, and despite prevailing tradewinds, is possible via equatorial countercurrents.

### *The Australasia to Africa scenario*

The one-parameter biogeographic model prefers dispersal from Australasia into Africa 12.3–7.8 mya, followed by trans-Atlantic dispersal to the New World 8.3–5.3 mya (Fig. 2B). The reconstruction under the DEC model supports a vicariant history in which the ancestor of *Crocodylus* was distributed across Australasia and Africa for approximately 10–15 my, with the lineage descending from the basal node leading to *C. mindorensis*, *C. novaeguineae*, and *C. johnstoni* inheriting an Australasian distribution. The lineage leading to the rest of *Crocodylus* maintained the broad African-Australasian distribution for approximately one million years, until a vicariant split between the African + Neotropical species clade and the remaining Australasian species, 12.3–7.8 mya (Fig. 2D). Such a prolonged, broad distribution and recent vicariance seems untenable given the timeframe of the *Crocodylus* radiation, and likely reflects the DEC model's tendency toward vicariant range inheritance; Ree and Smith (2008) and Clark et al. (2008) also found that the DEC model reconstructed vicariant divergence events in insular systems most likely limited to dispersal-mediated range evolution.

If we assume that Australasia is the origin of the MRCA, as strongly supported by the other three models and the constraint tests under the DEC model (Fig. 2 and Table 2), then the DEC model suggests dispersal from Australasia into Africa 12.3–7.8 mya leading to the African + Neotropical species lineage, which is congruent with the one-parameter model. Biogeographic analyses of amphibians in the Old World support the movement of four groups of frogs between Australasia and Africa at approximately the same time. Genetic data suggest that *Hoplobatrachus*, *Hylarana*, and *Chiromantis* invaded Africa from Asia (Kosuch et al. 2001), and *Duttaphrynus* moved between South Asia and the Arabian Peninsula (Van Bocxlaer et al. 2009), all during the late Miocene.

Evidence for the Australasia→Africa→Neotropics scenario also can be found in the crocodylian fossil record. Crocodylians were present in Southern Europe and Northern Africa during the Late Miocene, including possible *Crocodylus* (Delfino et al. 2007; Delfino and Rook 2008). Despite the ambiguity of *Crocodylus* being represented, these fossils nonetheless suggest that the warmer and wetter climate across this region during this period (Griffin, 2002) may have allowed true crocodiles to move from Asia into Africa without requiring long-distance marine



dispersal. This movement may have been coincident with Tibetan uplift initiating the Asian monsoon climate (Molnar et al. 1993).

The fossil record of extinct species of *Crocodylus* does not help decipher between initial dispersal from Australasia westward to Africa or eastward to the New World. The only two extinct species supported by phylogenetic analyses to fall out with extant *Crocodylus* are the Australasian *C. palaeindicus* from the Late Miocene and African *C. anthropophagus* from the Pliocene (Brochu et al. 2010). The relationships of both these species to extant *Crocodylus* based on morphological data are ambiguous (Brochu et al. 2010).

### The preferred estimate

I prefer the estimate of the biogeographic history of *Crocodylus* under the reversible-jump model, which strongly supports an Australasian origin, but is ambiguous about whether dispersal proceeded to Africa or the New World (Fig. 2C). Reversible-jump is a model-averaging method that integrates over the number of transition-rate parameters during Markov chain Monte Carlo (MCMC), and, in so doing, incorporates uncertainty in model selection (Pagel and Meade 2006). This behavior is particularly appealing for these data, because there is uncertainty in selecting the best-fit model of character evolution (Table S3) and conflicting results between the selected models (Fig. 2A,B).

Support for the direction of movement out of Australasia by the other models might be due to violations of the assumptions of these models. The higher likelihood of the two-parameter model over the one-parameter model results primarily from the improved fit of placing state changes (Neotropical→African) on the longer terminal branches leading to the two *C. niloticus* species, rather than the shorter internal branches leading to the Africa + New World clade (Fig. 2A). This improved fit may be an artifact of an assumption made by the continuous-time Markov models implemented in BayesTraits; they assume state-changes are independent of cladogenesis. In this empirical case, it seems intuitive that state-changes (i.e., dispersal to a different region of the world) are likely to cause cladogenesis, violating this assumption. The reversible-jump model makes the same assumption that cladogenesis and character change are independent, but incorporates uncertainty in whether parameterizations similar to the one- and two-parameter models are more appropriate for these data.

The DEC model allows character states to change at speciation events by reconstructing range-inheritance scenarios at the internal nodes. However, daughter lineages must inherit their range from their parent, thus for range evolution to occur, the DEC model must allow ancestral lineages to occur across multiple states (areas). Because it must allow broad (multistate) distributions, and it treats dispersal to establish such distributions independently of cladogenesis, the DEC model can infer vicariant scenarios even if

they are untenable (Clark et al. 2008; Ree and Smith 2008); this appears to be the case here. It does not seem possible that ancestral *Crocodylus* maintained a broad African-Australasian distribution for 11–16 my (Fig. 2D), given the continents were near their current positions throughout the timeframe of the radiation of the genus.

### Comparison to the alligators

The only other extant group of crocodylians that span more than one of the biogeographic character states is the genus *Alligator*; *A. mississippiensis* is restricted to the Southeastern United States, whereas *A. sinensis* is restricted to China. My estimate of the timing of the divergence between these sister species ( $\approx 58$ –31 mya; Table 1) is much older than the minimum divergence based on the fossil record (15 mya; Brochu 1999). Given the relative intolerance of *Alligator* to salt water (Taplin and Grigg 1989) and the temperate climate at high latitudes by the Miocene, dispersal within the past 20 my seemed problematic (Markwick 1998; Brochu 1999, 2003). My results suggest an earlier crossing of Beringia by *Alligator* during the warmer climate of the Early Tertiary (Miller et al. 1987), as proposed by Brochu (1999). If *Crocodylus* did initially disperse from Australasia to the New World approximately 12.3–7.8 mya, a Beringian route also is possible. However, an overwater route of dispersal might be more likely when considering (1) the temperate climate at such high latitudes by the Late Miocene, (2) the physiological adaptations of the genus to salt water, and (3) the West Pacific distribution of *C. porosus*.

### SURVIVING EXTINCTION

My estimate of the time-calibrated species tree strongly supports that *Crocodylus* diversified and colonized the globe during a period when crocodylians underwent a massive extinction (Fig. 1A). Following the mid-Miocene climatic optimum, there was a precipitous decline in crocodylian diversity coincident with global cooling and glacial advancement (Markwick 1998). The number of genera is estimated to have dropped from approximately 26 to 8 during the Pliocene, which represents the highest per-genus crocodylian extinction rate over the last 100 million years (Markwick 1998). As a result, most extant crocodylians represent relicts of formerly successful pre-Pliocene lineages, both in terms of diversity and distribution. For example, a great diversity of Caimaninae, *Gavialis*-related taxa, *Tomistoma*-related taxa, *Osteolaemus*-related taxa, and the currently unrepresented Mekosuchinae vanish from the fossil record near the end of the Tertiary (Brochu 2003). Congruent with the dating results here, the true crocodiles do not appear in the fossil record until quite recently, and when they do, most are diagnosable to living species (Molnar 1979; Miller 1980; Tchernov 1986; Delfino and DeVos 2010). Most extinct species referred to as *Crocodylus* have been

shown by phylogenetic analyses to fall outside the genus (e.g., “*Crocodylus*” *robustus*, *lloydi*, *megarhinus* and *pigotti*; Brochu 2007; Brochu et al. 2010). With the ambiguous relationships of *C. bambolii* (now cf. *Crocodylus* sp.; Delfino and Rook 2008), the only extinct species that are supported as *Crocodylus* are *C. palaeindicus* and *C. anthropophagus* (Brochu et al. 2010). Hence, there is no evidence for a tremendous loss of diversity in *Crocodylus* at the end of the Tertiary. Rather, my results show that *Crocodylus* radiated and colonized the tropics after global cooling and crocodilian extinction began.

The true crocodiles possess a suite of adaptations that make them better suited for hyperosmotic environments than other crocodylians, and might help explain the success of *Crocodylus* during the extinction of their relatives. *Crocodylus* possess lingual salt-secreting glands (Taplin and Grigg 1981; Taplin et al. 1982; Taplin 1988; Taplin and Loveridge 1988), a heavily keratinized buccal epithelium (Taplin and Grigg 1989), and a highly adapted osmoregulatory cloaca (Pidcock et al. 1997). Additionally, crocodiles have a broad range of thermal independence in swimming efficiency, allowing animals to disperse at suboptimal body temperatures (Elsworth et al. 2003; Seebacher and James 2008). Owing to these adaptations, *Crocodylus* may have been more vagile than its relatives, and able to locate suitable habitat during the onset of global cooling; perhaps competition with the highly successful true crocodiles contributed to the fate of many extinct crocodilians.

## RELATIONSHIPS OUTSIDE CROCODYLINAЕ

Another example of contentious, lower-level relationships within Crocodylia involves the Neotropical caimans (Caimaninae). Some phylogenetic estimates support the monophyly of the genus *Caiman* (Densmore 1983; Poe 1996; White 1992; Brochu and Densmore 2000; White and Densmore 2000; Gatesy et al. 2003, 1993), whereas others nest *Melanosuchus* within *Caiman*, rendering it paraphyletic (Densmore 1983; Poe 1996; Brochu 1997, 1999; Gatesy et al. 2003, 2004, 1993). This study strongly supports the monophyly of the *Caiman* genus, showing that *Melanosuchus niger* is the sister of all three *Caiman* species (Fig. 1B). Additionally, there is support for the distinctiveness of *Caiman yacare* and *Caiman crocodilus*. This finding is important, because the former is often considered a subspecies of the latter (Medem 1981; Ross 1998).

The African dwarf crocodile, *O. tetraspis*, seems to represent two distinct species in this study (Fig. 1). This is interesting because this species currently is thought to consist of two subspecies, *O. t. tetraspis* and *O. t. osborni* (Ross 1998); the latter formerly was considered a full species (Inger 1948), and originally was described as a separate genus (Schmidt 1919). My finding is consistent with the recent work of Eaton et al. (2009), whose results also suggest that *Osteolaemus* is comprised of multiple species.

Given the CITES appendix I protected status of this genus (Ross 1998), I support the decision of Eaton et al. (2009) to elevate the divergent lineages of *Osteolaemus* to separate species, and urge the immediate recognition of this taxonomy for the sake of their future conservation.

## The Gavialis debate

Unquestionably, the most contentious issue regarding the systematics of Crocodylia has been the placement of *Gavialis*. At present, there is still resistance to the molecular placement of *Gavialis* as sister to *Tomistoma* in favor of the traditional placement of *Gavialis* as sister to all other extant crocodylians (e.g., Piras et al. 2010). However, the results presented here once again demonstrate the sister-group relationship of *Gavialis* and *Tomistoma* within Crocodylidae. This relationship is unambiguously supported by the 10-locus dataset under both coalescent-based and concatenated phylogenetic models (Fig. 1 and Table 1). I hope the phylogeny inferred herein will be viewed as a framework within which to explore morphological and developmental data to understand better the interesting evolution that has occurred within Gavialinae (e.g., Gatesy et al. 2003).

## Conclusions

A complex new interpretation of the evolutionary history of *Crocodylus* emerges from this study. My results suggest that the genus originated from an ancestor in the tropics of the Serravallian or Tortonian Indo-Pacific,  $\approx 13.6$ –8.3 mya, and rapidly radiated and dispersed around the globe during a dire period in crocodylian evolution. During its circumtropical colonization, the genus underwent multiple transoceanic dispersals, perhaps crossing both the Pacific and Atlantic Oceans. These results overturn the long-held paradigm that the genus originated in Africa, and dispel any notion that the true crocodiles are ancient “living fossils.”

Furthermore, it is clear that the true diversity within the genus is not accurately represented by current taxonomy. There are at least two species encompassed within the taxon *C. niloticus*, and the current taxonomic boundaries for the freshwater crocodiles of New Guinea and the Philippine islands may not accurately reflect their evolutionary history. This work demonstrates the need for fine-scale, intraspecific sampling across all species of *Crocodylus* to better understand the diversity within the genus. Given that many *Crocodylus* are listed as critically endangered by the IUCN, such work is urgent.

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## Supporting Information

The following supporting information is available for this article:

**Figure S1.** Approximate geographic distributions of all Crocodylinae (*Crocodylus* with specific epithet only) separated into (A) Australasian, (B) Neotropical, and (C) African.

**Table S1.** The taxonomy used throughout this work.

**Table S2.** List of all tissue samples used in this study.

**Table S3.** Biogeographic model selection in BayesTraits.

**Table S4.** Primers used in PCR and cycle sequence reactions.

Supporting Information may be found in the online version of this article.

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