

Are crocodiles really monophyletic?—Evidence for subdivisions from sequence and morphological data

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Abstract

Recently, the phylogenetic placement of the African slender snouted crocodile, *Crocodylus cataphractus*, has come under scrutiny and herein we address this issue using molecular and morphological techniques. Although it is often recognized as being a “basal” form, morphological studies have traditionally placed *C. cataphractus* within the genus *Crocodylus*, while molecular studies have suggested that *C. cataphractus* is very distinct from other *Crocodylus*. To address the relationship of this species to its congeners we have sequenced portions of two nuclear genes (*C-mos* 302 bp and *ODC* 294 bp), and two mitochondrial genes (*ND6-tRNA^{glu}-cytB* 347 bp and control region 457 bp). Analyses of these molecular datasets, both as individual gene sequences and as concatenated sequences, support the hypothesis that *C. cataphractus* is not a member of *Crocodylus* or *Osteolaemus*. Examination of 165 morphological characters supports and strengthens our resurrection of an historic genus, *Mecistops* (Gray 1844) for *cataphractus*.

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1. Introduction

The African slender-snouted crocodile, *Crocodylus cataphractus*, has long been a systematic enigma. In one of the earliest systematic treatments of what is now called Crocodylia, Gmelin (1789) indicated that the habitat for *Lacerta gangeticus* (now *Gavialis gangeticus*) included rivers in “Senegal Africae et Gangen Indiae.” *Gavialis* has been restricted to the Indian subcontinent throughout historical times, but it is clear from Gmelin’s diagnosis that *C. cataphractus*, the crocodylian from Senegal with an elongate, subcylindrical rostrum, would have fallen within

L. gangeticus, highlighting the morphological gulf between *C. cataphractus* and other *Crocodylus*, reinforcing the need for further systematic analysis.

Various members of the genus *Crocodylus* (the true crocodiles) have been included in a number of phylogenetic studies, but until recently, very little had been written about relationships within *Crocodylus*. That the name “*Crocodylus*” lacked a uniform meaning renders comparisons of different scenarios virtually impossible. Neontologists were necessarily restricted to the 12 recognized living species, but paleontologists assigned fossils ranging throughout the Cenozoic and into the Mesozoic (sometimes as old as the Albian stage of the Cretaceous, between 99 and 112 mya) to *Crocodylus* (Markwick, 1998; Steel, 1973). Explicit diagnoses not reliant on overall head shape were rarely used, and *Crocodylus* was often a default category that simply meant a fossil could not be unambiguously assigned to some other

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genus. Several authors have suggested that the African slender-snouted crocodile (*C. cataphractus*) is the sister taxon to a clade comprising all other members of this genus (Brochu, 1997, 2000; Densmore, 1983; Densmore and Owen, 1989; Gatesy et al., 2003; Gatesy et al., 2004; White and Densmore, 2000). Most of these studies have suffered from limited taxon sampling and/or few representative individuals from the species being compared. To date there have been few studies aimed specifically at the relationship of this species to its congeners and no molecular studies. Recently, Schmitz et al. (2003) in a study on genetic variation within the Nile crocodile, *C. niloticus*, suggested that *C. cataphractus* formed a relationship outside the remainder of *Crocodylus*. However, this portion of their study included just a single *C. cataphractus* sample, two dwarf African crocodile samples (*Osteolaemus tetraspis*) and only three of the eleven recognized extant species of *Crocodylus* (*C. cataphractus*, *C. jonstoni*, and *C. niloticus*).

Herein, using more thorough taxon sampling and much larger sample sizes (especially for the nuclear gene sequences), we report sequence comparisons from both coding and non-coding regions of two nuclear protein-coding genes and from two different regions of the mitochondrial genome (also representing both coding and non-coding sequences) specifically to assess the relationship of *C. cataphractus* to other members of *Crocodylus* and to *Osteolaemus*.

The two nuclear markers sequenced for this study are the proto-oncogene *C-mos* and the gene that codes for ornithine decarboxylase (ODC). *C-mos* is a single-copy gene slightly over 1000 bp in length, contains no introns and codes for a protein (C-mos) involved in oocyte maturation during meiotic metaphase II (Saint et al., 1998; Yew et al., 1993). Due to its relative high degree of conservation, this gene provided the resolution necessary to examine generic level relationships within the Crocodylia.

The *ODC* gene codes for a protein that catalyses the conversion of ornithine to putricine (Friesen et al., 1999) and is involved in the control of cell growth and division (Yao et al., 1995). Comprising some 12 exons and 11 introns, it has a transcription unit 6–8 kb in length. Friesen et al. (1999) characterized a series of PCR primers for this gene spanning a region from intron 6 through intron 8. However, they did not test the amplicons produced with these primers for phylogenetic signal. While the use of the *ODC* gene in phylogenetic analyses has been limited, it has been shown to be comparable to both mitochondrial cytochrome *b* (Allen and Omland, 2003) and control region (Kulikova et al., 2004) sequences at resolving phylogenetic relationships.

Mitochondrial sequence data continue to be widely used in many systematic studies, including crocodylians (Gatesy and Amato, 1992; Gatesy et al., 2003; Gatesy et al., 2004; Ray et al., 2000; Schmitz et al., 2003; White and Densmore, 2000). While most crocodylian mitochondrial datasets have focused on the region that includes the cytochrome *b* gene or the ribosomal DNA genes, we sequenced a region that includes *ND6-tRNA^{glu}-cytB* (*ND6-cytb*) genes as well as a portion of the mitochondrial control region. This choice

was largely based on recent studies (Ray and Densmore, 2002, 2003; White and Densmore, 2000), which indicate that these sequences are effective markers in the Crocodylia, especially for comparisons involving closely related taxa.

Morphological comparisons were naturally made between *C. cataphractus* and the other slender-snouted species of *Crocodylus* (*C. intermedius* and *C. jonstoni*), but it was generally agreed that these represented independent derivations of a specialized snout morphology (e.g., Meyer, 1984; Mook, 1921; Schmidt, 1924; Sill, 1968). Many slender-snouted crocodylians from throughout the Cenozoic have been referred either to *C. cataphractus* or a putative relative (e.g., Aoki, 1992; Pickford, 1994; Storrs, 2003; Tchernov, 1986), but assignments were often based on overall skull shape and not synapomorphy. Tchernov (1986) argued that the other extant African species of *Crocodylus* (the Nile crocodile *C. niloticus*) was closer to the Indian mugger (*C. palustris*) than to *C. cataphractus*. Based on fossils he presumed to be ancestral to living species, the last common ancestor between *C. cataphractus* and *C. niloticus* was no younger than the Late Eocene, but relationships with other non-African species were not discussed. Aoki (1976, 1992) went further, arguing that *C. cataphractus* was closer to the other living longirostrine crocodylians (*Gavialis* and *Tomistoma*) than to other living *Crocodylus*.

At present, the most comprehensive analyses of morphological data support *Crocodylus* monophyly, but nevertheless place *C. cataphractus* outside a clade including all other extant *Crocodylus* (Brochu, 2000). The vast majority of fossils previously assigned to *Crocodylus* do not belong to the crown genus. Recent work has shown that most fossil *Crocodylus* from the African Neogene are actually closer to *Osteolaemus* (Brochu, 2003, in review). The oldest fossils unambiguously falling within the crown genus are from the Middle Miocene, which is consistent with suggestions from molecular data that *Crocodylus* is a geologically young radiation (Densmore, 1983; Hass et al., 1992). However, morphological support for relationships within *Crocodylus* is comparatively weak, reflecting the emphasis placed on osteological characters by most morphological analyses. Skeletal evidence for deeper crocodylian nodes is extensive, but shallower species-level divergences throughout the clade tend to be supported by more subtle characters, and nodal support tends to be low.

Herein, we provide genetic and morphological evidence for the resurrection of a historical generic name *Mecistops* (Gray 1844) for *C. cataphractus*. As such, *C. cataphractus* will be recognized throughout the remainder of this manuscript as *M. cataphractus*.

2. Molecular methods

2.1. Blood collection and DNA extraction

Whole blood was collected from either the ventral caudal sinus (Gorzula et al., 1976) or the dorsal postcranial sinus (Bayliss, 1987) and used as the source of DNA for this

study. All blood samples are maintained in the frozen tissue collection at Texas Tech University by LDD. DNA extractions for all individuals included in this study were performed using a PureGene DNA extraction kit (Minneapolis, MN) with modifications.

2.2. Sampling protocols

2.2.1. *C-mos* sequences

Forty-nine individual DNA samples were analyzed in this study representing all eight extant crocodylian genera. Twenty-one individual *Crocodylus* (10 species), six *M. cataphractus*, three *Alligator mississippiensis*, two *A. sinensis*, six *Osteolaemus tetraspis*, three *Caiman yacare*, one *Melanosuchus niger*, one *Paleosuchus trigonatus*, two *Tomistoma schlegelii*, and four *Gavialis gangeticus* were included in this dataset. GenBank accession numbers are AY910583–AY910626 for samples examined in this project. Five samples were downloaded from NCBI (Accession Nos. AF039484, AF478194–478196, and AY447979,) and used in analyses.

2.2.2. *ODC* sequences

Twenty individual crocodylian DNA samples were amplified and sequenced for the nuclear gene *ODC* representing all eight extant genera: 10 *Crocodylus* (seven species), four *M. cataphractus*, one *A. mississippiensis*, one *A. sinensis*, one *G. gangeticus*, one *T. schlegelii*, and two *O. tetraspis*. GenBank accession numbers are AY914746–AY914765.

2.3. PCR amplification of nuclear genes

Polymerase chain reaction (PCR) amplifications were performed using *Thermus aquaticus* DNA polymerase (Saiki et al., 1986, 1988) in reaction volumes of 50 μ l following protocols described by Allard et al. (1991). PCR was performed using gene-specific primers (Table 1) and an Eppendorf Mastercycler gradient thermocycler (Brinkmann Instruments, Westbury, NY). Each reaction consisted of 3 μ l of each primer (10 μ M), 3 μ l $MgCl_2$ (25 mM), 2 μ l of dNTP (1 mM each), 2.5 μ l of *Taq* (5 U/ μ l), 5 μ l of 10 \times buffer, and dH₂O to a final volume of 50 μ l. The volume of template added to each reaction (Table 1) varied according to the gene amplified. Amplification began with an initial denaturing step of 95 °C for 2 min and 25 cycles were then performed with the following parameters: 45-s denaturation at 95 °C; 30-s annealing at appropriate temperature (Table 1); and a 50-s extension at 72 °C. Amplification ended with a 10 min extension step of 72 °C followed by a 4 °C hold. Polymerase chain reactions were purified using

the Qiagen PCR purification kit (Qiagen, Valencia, CA) following the protocol outlined in the supplied handbook.

2.3.1. *ND6-tRNA^{glu}-cytB* sequences

Sequence data from over 70 animals representing all species were originally collected by White (1992). From these samples, amplicons that yielded the highest quality sequence data were ultimately used in the current analyses, representing a single individual from every extant species of Crocodylia. GenBank accession numbers are AY914766–AY914788.

2.3.2. Mitochondrial control region sequences

Seventeen aligned sequences were previously used in Ray and Densmore (2002), Accession Nos. AF460206–AF460218, AF461417, Y13113, AJ404872, and NC004448. Tandemly repeated motifs occurred in the 3' end of the control region in all taxa; thus only non-tandemly repeated sequences were used in the phylogenetic analyses.

PCR amplification of mitochondrial sequences was accomplished in two ways. For the *ND6-tRNA^{glu}-cytB* sequences, primers CB2H, CB2Hint, ND5L2, and ND6L were employed (see Ray et al., 2000; White, 1992 for details). For the mitochondrial control region sequences, the primers CR2H (5'-GGG GCC ACT AAA AAC TGG GGG-3') and tPhe-L (5'-GAA CCA AAT CAG TCA TCG TAG CTT AAC-3') were used for all but *M. cataphractus* and members of the Alligatoridae. Amplification of *M. cataphractus* was accomplished using CR2H and 17774L (Quinn and Mindell, 1996). Control region sequences for *A. mississippiensis* (Janke and Arnason, 1997) and *Caiman crocodylus* (Janke et al., 2001) were obtained directly from GenBank (Accession Nos. Y13113 and AJ404872, respectively).

Mitochondrial sequence amplification protocols were similar to the above nuclear gene amplification protocols with specific adaptations as described in Ray and Densmore (2002) for the control region sequences and a modified “touchdown” PCR protocol (Don et al., 1991) was used for the ND6-cytb region (White, 1992).

2.4. Cycle or manual DNA sequencing

Purified PCR products were sequenced using either an ABI 310 or an ABI 3100 automated sequencer (Perkin-Elmer, Foster City, CA), ABI Big Dye chemistry (Perkin-Elmer, Foster City, CA), and the amplification primers (*C-mos*, *ODC*, and mitochondrial control region sequences). Cycle sequencing and purification of sequencing products were performed following the standard guidelines of Perkin-Elmer for BIG DYE v. 3.1. Samples were then pre-

Table 1
Primers, DNA template added and annealing temperature for PCRs of nuclear encoded genes used in this study

	Primers	Volume of template (ng)	Annealing temperature (°C)
<i>C-mos</i>	CMOS-77 and CMOS-78 or CMOS-74 and CMOS-78 Saint et al. (1998)	50 to 100	55
<i>ODC</i>	ODE-6 and ODE-8 Friesen et al. (1999)	75 to 150	54

pared for sequencing following Perkin-Elmer guidelines with a running time of 34 min per sample. The *ND6-tRNA^{glu}-cytB* sequences were determined manually using a modified Sanger–Coulson dideoxy chain termination protocol as described by Palumbi et al. (1991). Additional technical details are presented in White (1992).

2.5. Data analyses

Sequences were aligned using Vector NTI Suite software version 7.0 (Informax Inc., 2000) and verified by eye. The aligned sequences were then analyzed using PAUP v. 4.0b10 (Swofford, 2002) and Mr. BAYES v3.0 (Huelsenbeck and Ronquist, 2001). *Alligator mississippiensis* was used as the outgroup taxon in all analyses. Gene sequences were analyzed individually and as concatenated sequences using maximum-likelihood and Bayesian methods. Analyses were then compared for congruence of relationships. Sequence divergence values were calculated using uncorrected pairwise values. In maximum-likelihood analyses, Modeltest (Posada and Crandall, 1998) was used to determine the most appropriate evolutionary model for each of our datasets. Parameters were then set to the most appropriate model for the dataset (*C-mos*—K2P+I+G (Kimura, 1980); *ODC*—K81+G (Kimura, 1981); *ND6-tRNA^{glu}-cytB* region and mitochondrial control region—TrN+G (Tamura and Nei, 1993)).

Bayesian analyses were performed using MR. BAYES v3.0 (Huelsenbeck and Ronquist, 2001). MrModeltest (Nylander, 2004) was used to determine the most appropriate evolutionary model for each dataset. Models chosen varied by dataset (*C-mos*—K2P+G; *ODC*—K81+G; *ND6-cytb* region and mitochondrial control region—GTR+G (Lanave et al., 1984; Rodriguez et al., 1990)). To evaluate the parameters used, a metropolis-coupled MCMC was run with six incremental chains. A starting tree was chosen at random, 10.0×10^6 generations were run, with sampling every 100 generations using the most appropriate model of evolution for the dataset. In all searches, stationarity of the Markov chain was determined as the point when sampled log-likelihood values plotted against generation time reached a stable value. A burnin of 5000 trees was then set producing 95,001 sample points. The resulting trees were used to generate a majority consensus

tree with posterior probability values. Nodes with values of 85–89 were considered to have low support, 90–94 to have moderate support and nodes greater than 95 to be highly supported (Huelsenbeck and Ronquist, 2001). In the case of concatenated datasets, we chose to utilize a complex model of evolution GTR+I+G (Lanave et al., 1984; Rodriguez et al., 1990). This model was chosen a priori allowing us to test observed relationships developed utilizing best fit models for individual gene sequences to a randomly chosen model of evolution. Our rationale was that if relationships remain consistent across models of evolution, you are most likely retrieving the correct evolutionary relationships and not the effects of an evolutionary model on the dataset.

3. Morphological methods

3.1. Institutional abbreviations

TMM, Texas Memorial Museum, Austin, TX; UCMP, University of California Museum of Paleontology, Berkeley; UF, Florida Museum of Natural History, Gainesville.

The morphological analysis was based on a matrix of 165 characters and 60 ingroup taxa (Brochu, 1999, 2004a, 2006, in review; Appendix A). The analysis included all extant crocodylids and *Gavialis*, and two species of *Osteolaemus* (*O. tetraspis* and *O. osborni*) were recognized. Alligatoroid sampling was restricted to reduce computational time. Trees were rooted on two fossil outgroups (*Bernissartia fagesii* and *Hylaeochampsia vectiana*).

Two different maximum parsimony analyses were undertaken—one with taxon relationships unconstrained (beyond outgroup designation), and another in which *Mecistops* was constrained to fall closer to *Osteolaemus* and its extinct relatives (the osteolaemines, Appendix A). In both cases, 100 random-seed heuristic searches were completed using PAUP v. 4.0b10 (Swofford, 2002).

4. Results

4.1. *C-mos*

A total of 528 bp of *C-mos* sequence data was collected for most individuals. Due to missing sequence in several

Table 2

Uncorrected pairwise genetic distance values for the nuclear genes *C-mos* and *ODC* as well as the ND6 and control regions of the mitochondrial genome

	<i>C-mos</i> (%)	<i>ODC</i> (%)	ND6 region (%)	Control region (%)
Within <i>M. cataphractus</i>	0.70	0.18		
<i>M. cataphractus</i> to <i>Crocodylus</i>	1.80	1.62	17.09	14.51
<i>M. cataphractus</i> to <i>Osteolaemus tetraspis</i>	1.34	3.43	19.10	14.10
<i>M. cataphractus</i> to Gharials	2.40	6.47	22.89	12.79
Within <i>O. tetraspis</i>	0.22	1.05	8.84	
<i>O. tetraspis</i> to <i>Crocodylus</i>	0.81	2.71	20.23	14.52
Within <i>Crocodylus</i>	0.94	0.61	8.08	10.47
<i>Crocodylus</i> to Gharials	2.83	5.73	22.94	14.25
<i>Gavialis gangeticus</i> to <i>Tomistoma schlegelii</i>	0.41	1.70	22.36	18.79
<i>Alligator mississippiensis</i> to <i>A. sinensis</i>	0.99	3.31	15.30	15.91
Within Caimans	0.00	0.79	10.58	

individuals at the 3' end of this region, a total of 302 bp were analyzed for all species in this study. Intraspecific sequence variation within this marker was minimal and provided little or no resolution below the species level. However, intrageneric variation was moderate, consisting primarily of indels ranging from 1 to 3 bp in size. In no case did these events lead to stop codons within the reading frame for this marker; however, we must note that we sequenced only a portion of the CMOS gene and as such

are unable to determine the effects these indels may have on the structure of the protein. Insertion/deletion events, while uncommon in protein coding genes, have been reported to occur within this gene sequence in songbirds (Lovette and Bermingham, 2000) and two snake families (Saint et al., 1998). Of the 302 sites examined, there were 272 constant sites, 5 parsimony uninformative sites, and 25 parsimony informative sites. High conservation and a lack of introns (often the location of most variation in nuclear genes)

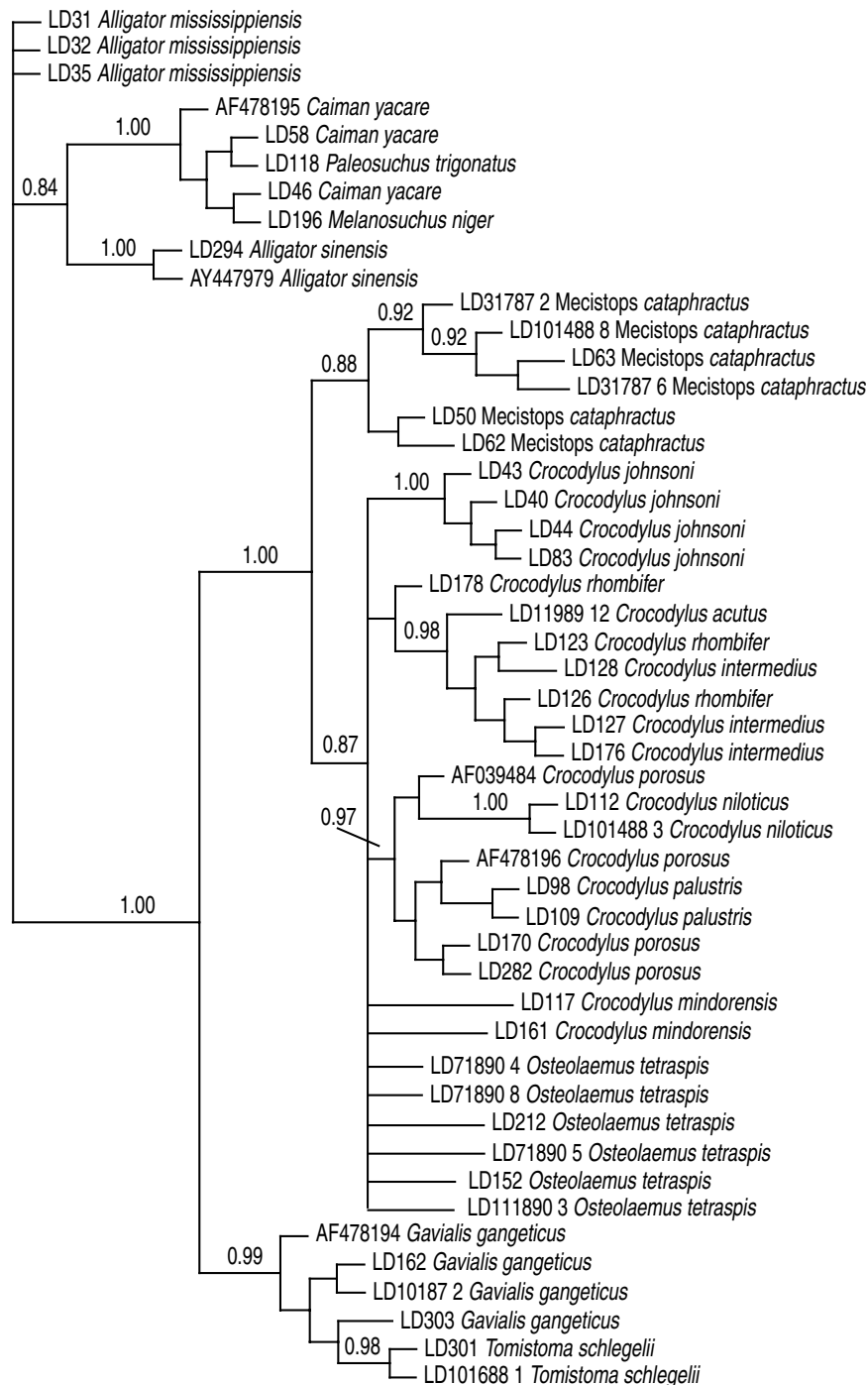


Fig. 1. Consensus Bayesian tree illustrating the relationships of crocodylians using the evolutionary model of K2P + G (Kimura, 1980) and sequences from the nuclear gene *C-mos*. Starting tree was chosen at random and 10.0×10^6 generations run with sampling every 100 generations and a burnin of 5000 resulting in 95,001 sample points. Values above the nodes are Bayesian posterior probability values.

produced relatively low genetic distance values within this dataset. However, it is interesting to note the relatedness between *M. cataphractus*, *O. tetraspis*, and the remaining *Crocodylus* species; genetic distance values are lower between *Osteolaemus* and all other species of *Crocodylus* than the genetic distances between *M. cataphractus* and the remaining members of *Crocodylus* (Table 2).

While there have been a limited number of systematic studies using *C-mos*, these have primarily been at higher taxonomic levels (Cooper and Penny, 1997; Saint et al.,

1998). In our study, at least at the generic level, *C-mos* provided adequate resolution. maximum-likelihood and Bayesian analyses produced trees with essentially identical topologies; only the Bayesian tree is shown here (Fig. 1). Bayesian posterior probability values provide weak to strong support at most major nodes (i.e., at the generic level, Fig. 1) but not among more closely related species.

This marker corroborates several relationships that have been historically supported. In our analyses all new world true crocodiles are united in a single clade with strong

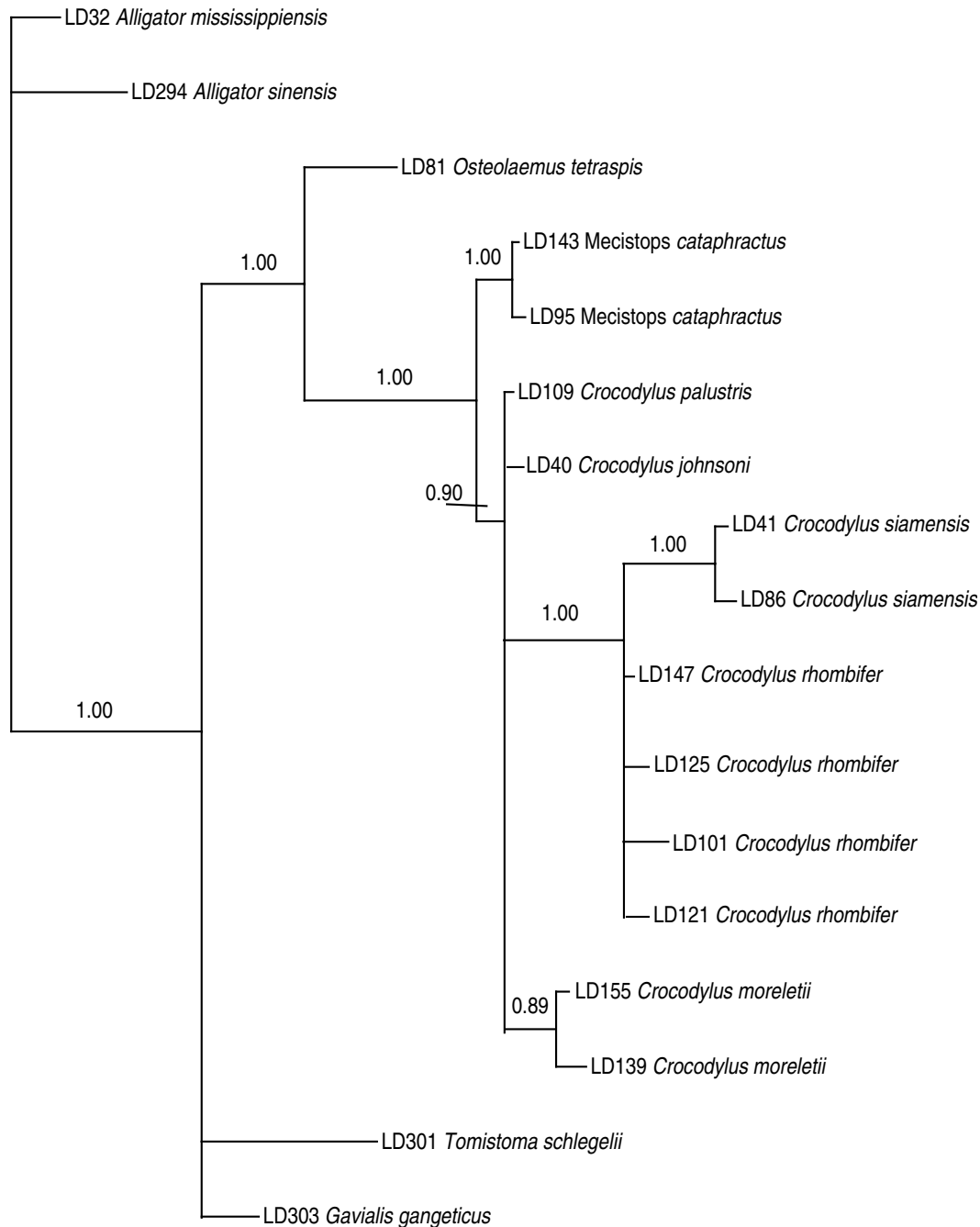


Fig. 2. Consensus Bayesian tree illustrating the relationships of crocodylians using the evolutionary model of K81 + G (Kimura, 1981) and sequences from the nuclear gene *Ornithine-decarboxylase*. Starting tree was chosen at random and 10.0×10^6 generations run with sampling every 100 generations and a burnin of 5000 resulting in 95,001 sample points. Values above the nodes are Bayesian posterior probability values.

Bayesian support. Unlike the New World *Crocodylus*, Old World *Crocodylus* does not form a clade and has virtually no support for the recovered relationships. *Mecistops cataphractus* maintains a sister-taxon relationship to a clade comprising all remaining *Crocodylus* and *Osteoleaemus*, though with low support values (Fig. 1).

4.2. ODC

Two hundred ninety-four basepair of sequence from both introns and exons for the nuclear gene *ODC* showed several indels ranging from 1 to 3 bp in length. Of 294 characters analyzed, 211 were constant, 59 were parsimony

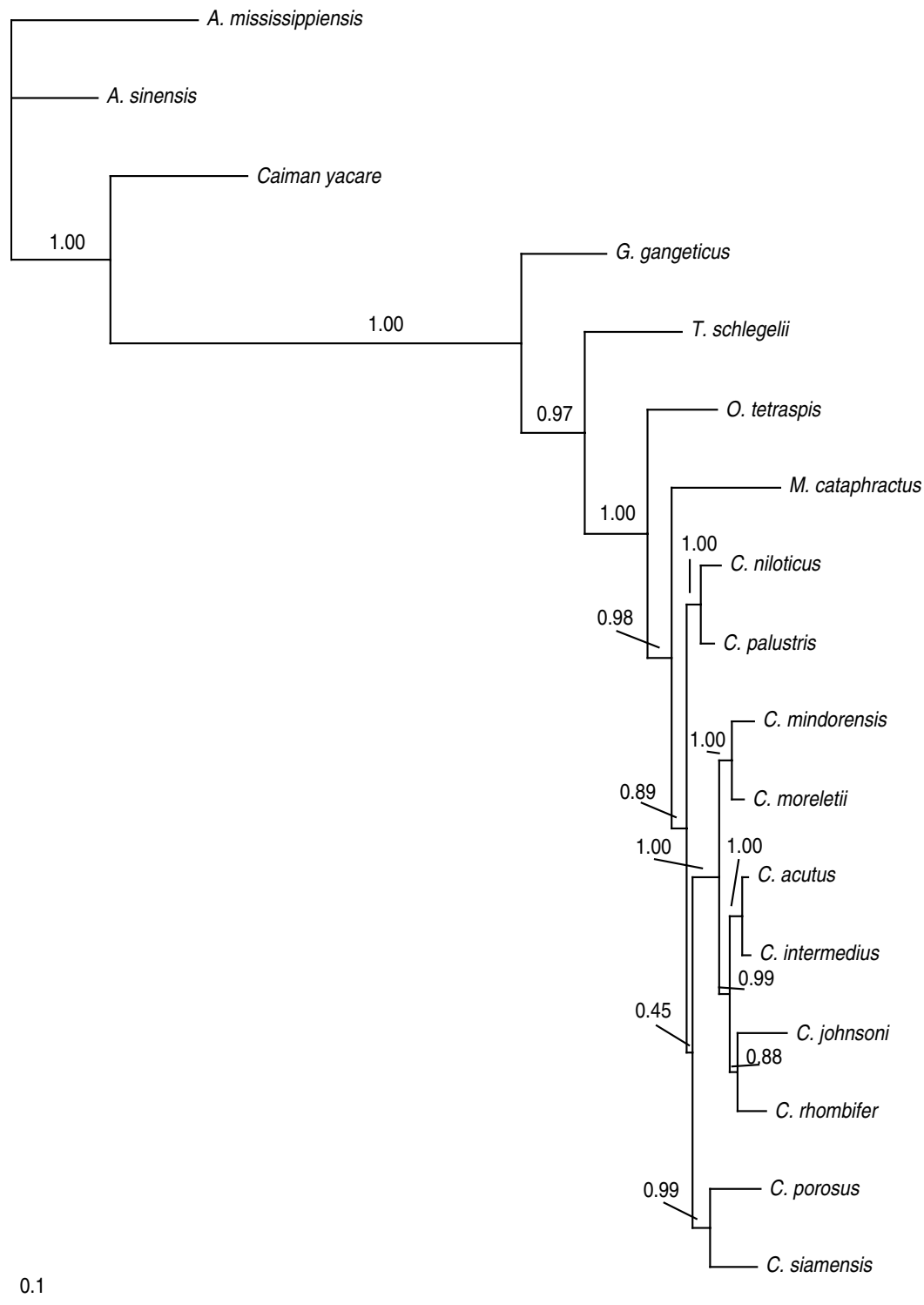


Fig. 3. Consensus Bayesian tree illustrating the relationships of crocodylians using the evolutionary model of GTR + G (Lanave et al., 1984; Rodriguez et al., 1990) and sequences from the ND6 region of the mitochondrial genome. Starting tree was chosen at random and 10.0×10^6 generations run with sampling every 100 generations and a burnin of 5000 resulting in 95,001 sample points. Values above the nodes are Bayesian posterior probability values.

uninformative, and 24 were parsimony informative. Analysis of genetic distance values provided little resolution at the interspecific level. Intergeneric relationships in Bayesian and ML analyses are consistent with those seen in other molecular datasets. *ODC* analyses group *M. cataphractus* with a clade that contains *Osteolaemus* and *Crocodylus* (Fig. 2).

4.3. Mitochondrial *ND6-tRNA^{glu}-cytB* region

Analysis of 347 bp of *ND6-cytb* sequence resulted in 150 constant characters, 45 parsimony uninformative characters, and 152 parsimony informative characters. Genetic distance values within this dataset, while higher, have pat-

terns similar to our nuclear datasets (Table 2). Genetic distance values for this dataset ranged from a low of 8.08% within the genus *Crocodylus* to a high of 22.94% between *Crocodylus* and the true and false gharials. Within genera, *ND6-cytb* values ranged from 8.08% within *Crocodylus* to 8.84% within *O. tetraspis*, values well below the 17.09% between *M. cataphractus* and *Crocodylus*.

Due to the similarity between the analyses, only the Bayesian tree is shown here (Fig. 3). As can be readily seen, *ND6-cytb* sequence comparisons produced relationships similar to those seen in our nuclear datasets with *M. cataphractus* sister to a clade containing the remaining members of the genus *Crocodylus* (Fig. 3). The comparatively high support for *M. cataphractus* being the sister-

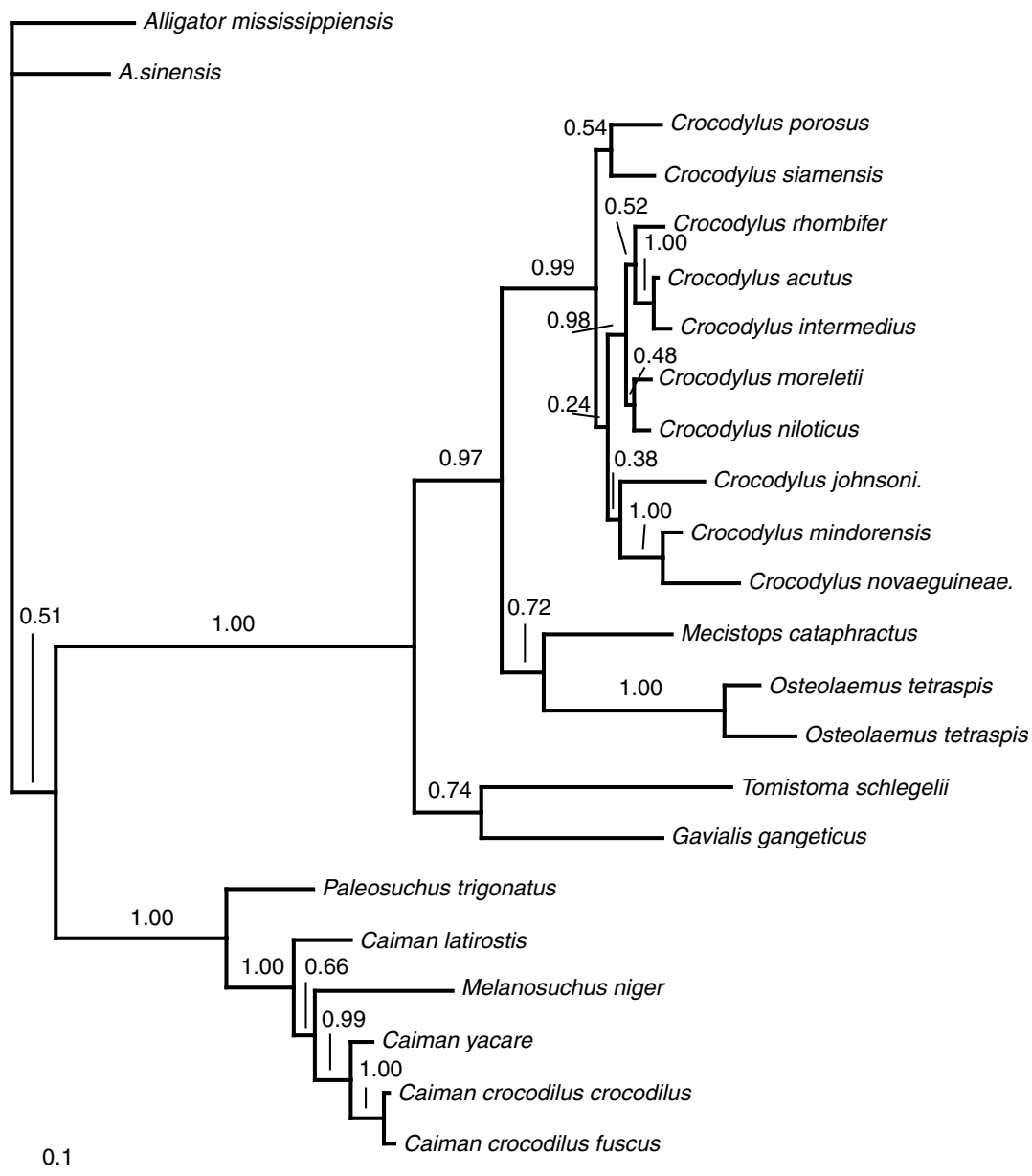


Fig. 4. Consensus Bayesian tree illustrating the relationships of crocodylians using the evolutionary model of GTR + G (Lanave et al., 1984; Rodriguez et al., 1990) and sequences from the control region of the mitochondrial genome. Starting tree was chosen at random and 10.0×10^6 generations run with sampling every 100 generations and a burnin of 5000 resulting in 95,001 sample points. Values above the nodes are Bayesian posterior probability values.

taxon to the remaining *Crocodylus* is most likely due to an increased rate of mutation within the *ND6-cytb* region of the genome as compared to our nuclear datasets.

4.4. Mitochondrial

4.4.1. Mitochondrial control region

Within portions of these mitochondrial sequences, there appears to be an increased rate of mutation. This is evident in comparisons of uncorrected pair-wise genetic distance

values (Table 2). Values for this region of sequence ranged from a low of 10.47% within *Crocodylus* to a high of 18.79% between the two gharials. As noted for *C-mos* and *ODC* analyses, genetic distance values are lower between *Crocodylus* and *Osteolaemus* (Table 2) than between *Crocodylus* and *M. cataphractus* (Table 2). A comparison between interspecific distance values of “other” *Crocodylus* and *M. cataphractus* also support the exclusion of *M. cataphractus* from *Crocodylus* (Table 2). While interspecific genetic distance values in this dataset for this region are relatively

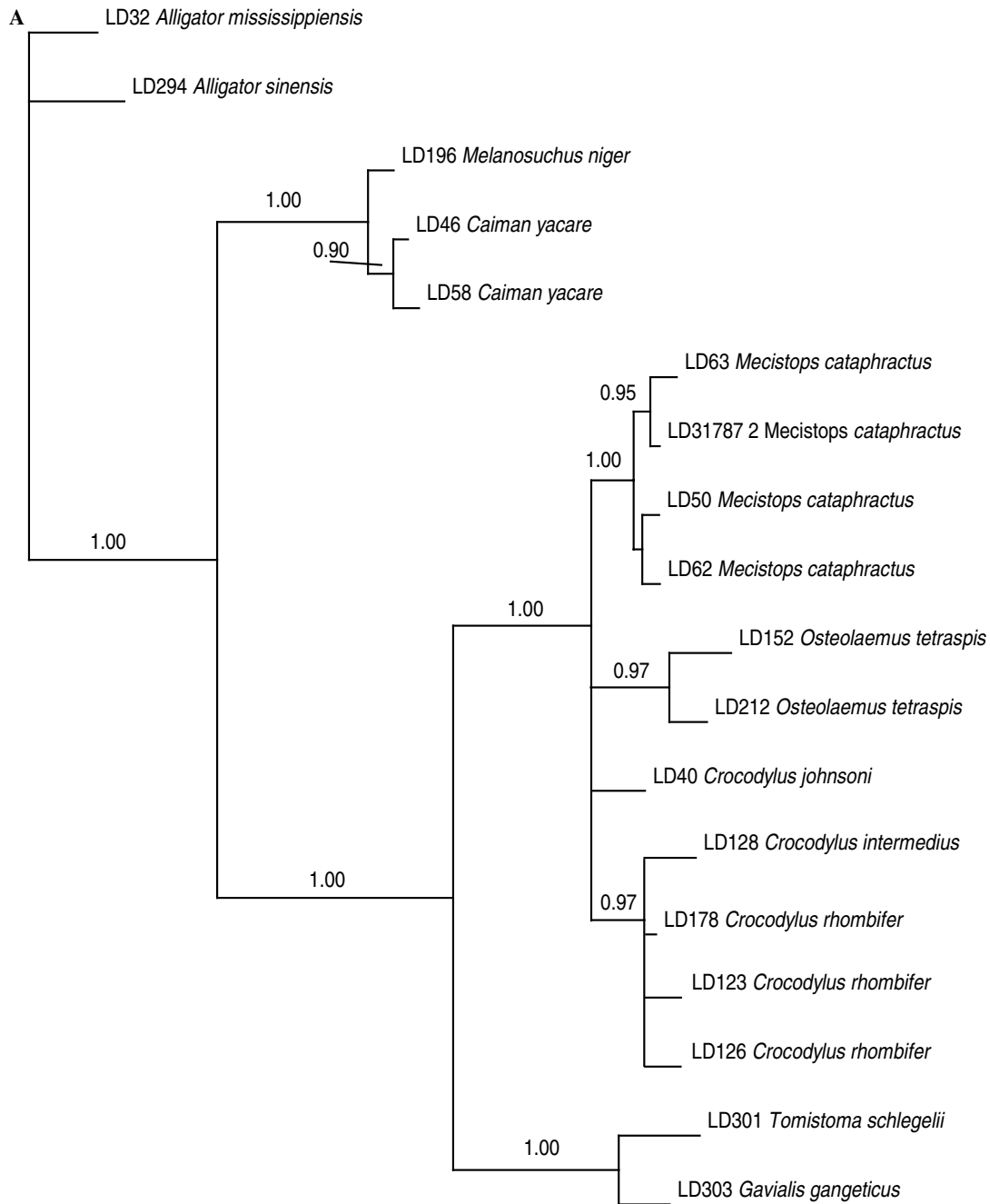


Fig. 5. Consensus Bayesian trees illustrating the relationships of crocodylians using the evolutionary model of GTR +I + G (Lanave et al., 1984; Rodriguez et al., 1990) and sequences from the *C-mos* and *ODC* genes (tree A) and the mitochondrial control region and ND6 regions (tree B). Starting tree was chosen at random and 10.0×10^6 generations run with sampling every 100 generations and a burnin of 5000 resulting in 95,001 sample points. Values above the nodes are Bayesian posterior probability values.

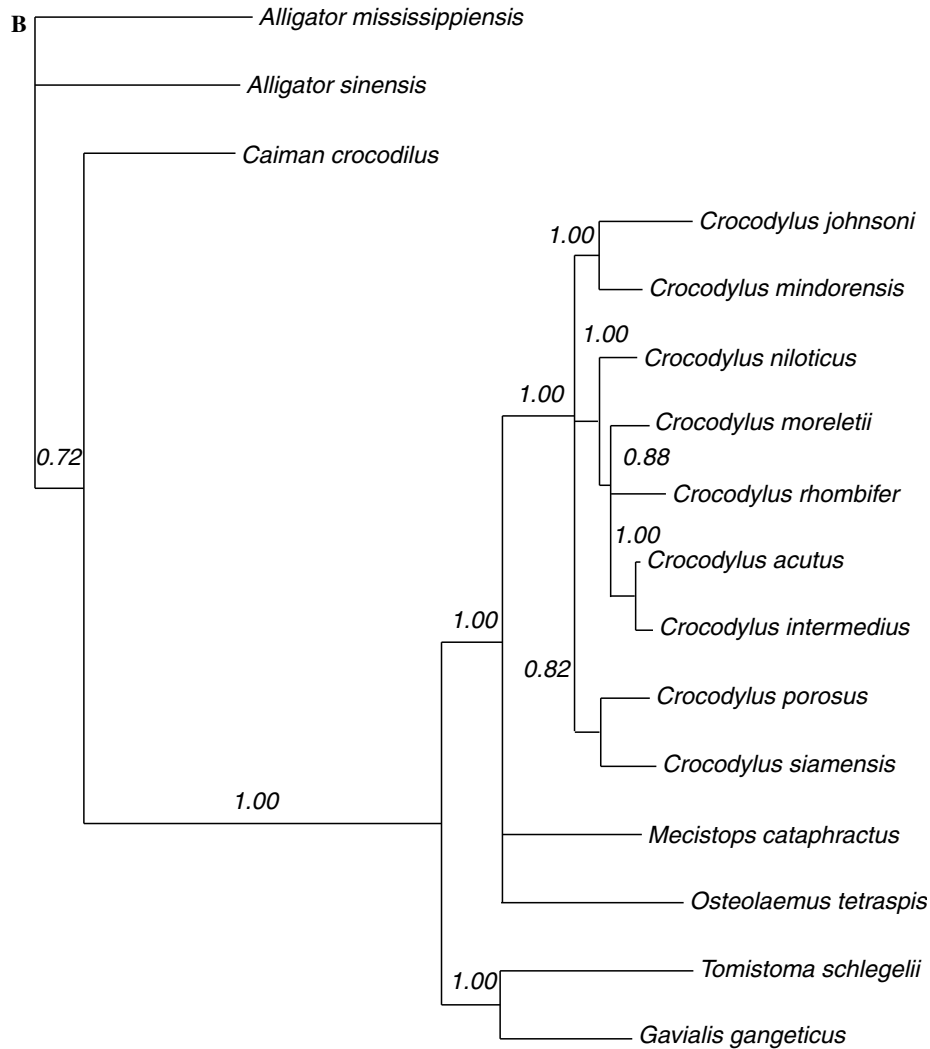


Fig. 5. (continued).

high, they are certainly well within values reported in studies of rattlesnakes (Ashton and de Queiroz, 2001), rodents (Castro-Campillo et al., 1999), and crocodiles (Ray et al., 2004). As with *C-mos*, *ODC*, and *ND6-cytb* sequence analyses, control region ML and Bayesian analyses produced trees with nearly identical topologies. Relationships between *Osteolaemus*, *M. cataphractus*, and *Crocodylus* are consistent with those seen in our other three datasets. However, unlike the other datasets, control region sequences place *M. cataphractus* and *O. tetraspis* as sister taxa albeit with low support (Fig. 4).

4.4.2. Concatenated sequences

Studies have shown that in cases where there are few informative characters, concatenation of datasets can increase support for relationships as well as increase the likelihood of generating the true phylogenetic tree (Flynn et al., 2005; Gadagkar et al., 2005). Due to differences in numbers of individuals sequenced, we developed two concatenated datasets, one for nuclear gene sequences

(Fig. 5A) and one for mitochondrial gene sequences (Fig. 5B). Maximum-likelihood and Bayesian analyses of these datasets are consistent with our analyses of individual genes; however, nodal support values are consistently higher (Figs. 5A and B).

4.5. Morphological data

4.5.1. Unconstrained morphological analysis

The unconstrained analysis produced 38,134 equally optimal trees (Fig. 6). As in previous analyses of morphology, four characters unambiguously linked *Mecistops* with *Crocodylus* (Brochu, 2000): a “wasp-waisted” ilium in which the iliac blade is deeply notched dorsally near its posterior tip; a truncated surangular that does not extend to the dorsal tip of the posterolateral wall of the glenoid fossa; a deeply forked anterior ectopterygoid ramus; and a series of blind pockets on the medial wall of the caviconchal recess of the maxilla (Fig. 7). In addition, the surangular–angular suture passes along the posteroventral margin of

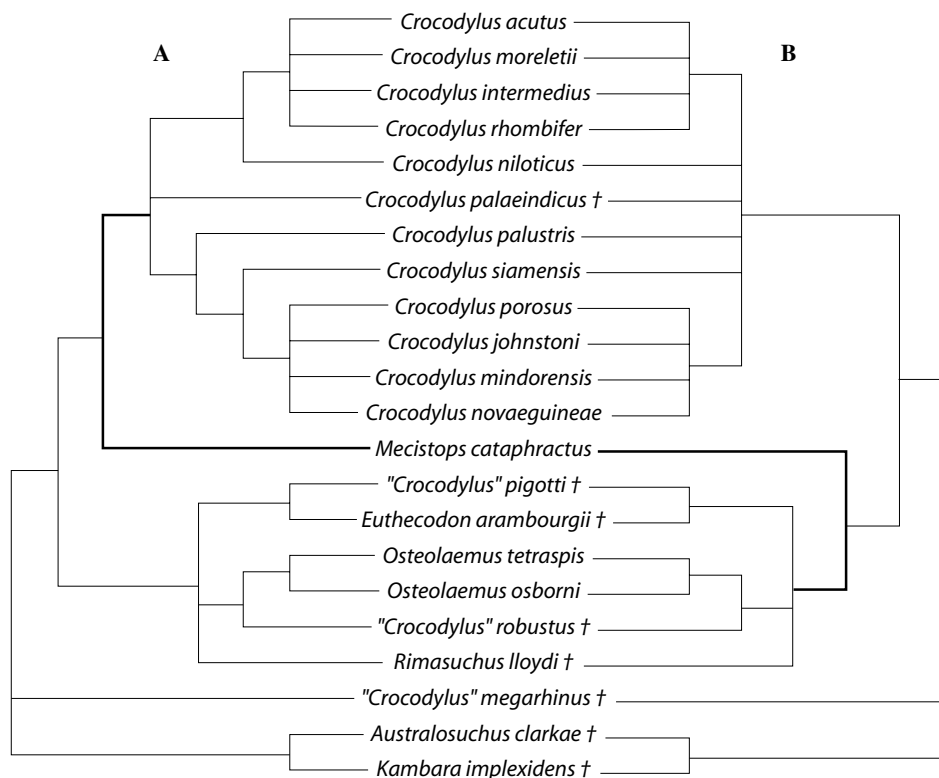


Fig. 6. Relationships among crocodylids based on analysis of morphological data. Complete list of ingroup taxa in Appendix A; see Brochu (2004a, 2006) for details on relationships outside Crocodylidae. (A) Unconstrained analysis (strict consensus of 38,313 equally optimal trees, length = 448, CI excluding uninformative characters = 0.423). (B) Analysis constrained to draw *Mecistops* closer to *Osteolaemus* (strict consensus of 449,339 equally optimal trees, length = 453, CI excluding uninformative characters = 0.418). † = extinct.

the external mandibular fenestra in *Mecistops* and *Crocodylus* (Norell, 1989), but this character state has a complex distribution in other crocodylids—the derived condition is also found in *O. tetraspis* and *T. schlegelii*, but the plesiomorphic condition (in which the suture simply terminates at the posterior end of the fenestra) is found in all other tomistomines, the osteolaemines, “*Crocodylus*” *robustus* and *O. osborni* (Brochu, in review), mekosuchines, and “*Crocodylus*” *megarhinus*. It thus might represent multiple derivations or losses among crocodylids, including *Crocodylus* and *Mecistops*.

One robust unambiguous synapomorphy unites *Crocodylus* to the exclusion of *Mecistops*. In *Mecistops* (as in most other crocodylians), the lateral eustachian foramina open lateral and dorsal to the larger median eustachian foramen at maturity (Fig. 8A). The mature condition in *Crocodylus* resembles what is seen in all crocodylians early in ontogeny—the lateral eustachian foramen is almost directly lateral to its medial counterpart (Fig. 8B). In part, this reflects a trend seen among crocodylids generally—a shortening of the midline concavity of the pterygoid against which the sheetlike posterior lamina of the basisphenoid lies—but the lateral eustachian openings still shift dorsally to a greater extent in *Mecistops*, *Osteolaemus*, and *Tomistoma* (Brochu, 2000).

A closer look at one of these characters raises questions about homology. Previously, in the absence of a hemi-

sected or disarticulated skull, the condition of the caviconchal recess could only be assessed by looking obliquely through the external naris or adductor chamber, which made accurate analysis problematic. New high-resolution computed tomographic (CT) images for *Mecistops* (Fig. 7C) reveal deep pockets along the medial surface of the caviconchal recess—the blind “caecal recesses” used to diagnose *Crocodylus* (including *Mecistops*) by Brochu (2000). Like other pneumatic features among archosaurs, expression of these pockets can vary within species (Witmer, 1995), but they are not observed in other crocodylians. However, CT images for extant longirostrine crocodylians, such as *Tomistoma* (Fig. 7), indicate a similar series of concavities on the surface of the caviconchal recess. Like the features seen in *Mecistops*, but unlike those of *Crocodylus*, these correspond precisely to maxillary alveolar positions and are uniformly large. A similar phenomenon is seen in *Gavialis* (Brochu, pers. obs.), and we might be seeing a consequence of snout shape—as the rostrum becomes narrow, the medial wall of the caviconchal recess might begin to reflect the positions of alveoli that would otherwise not be expressed so far medially. Further study is required to test the distribution of these features in other longirostrine crocodylians and whether the structures seen in the single specimen of *Mecistops* submitted for CT analysis are a species-wide phenomenon.

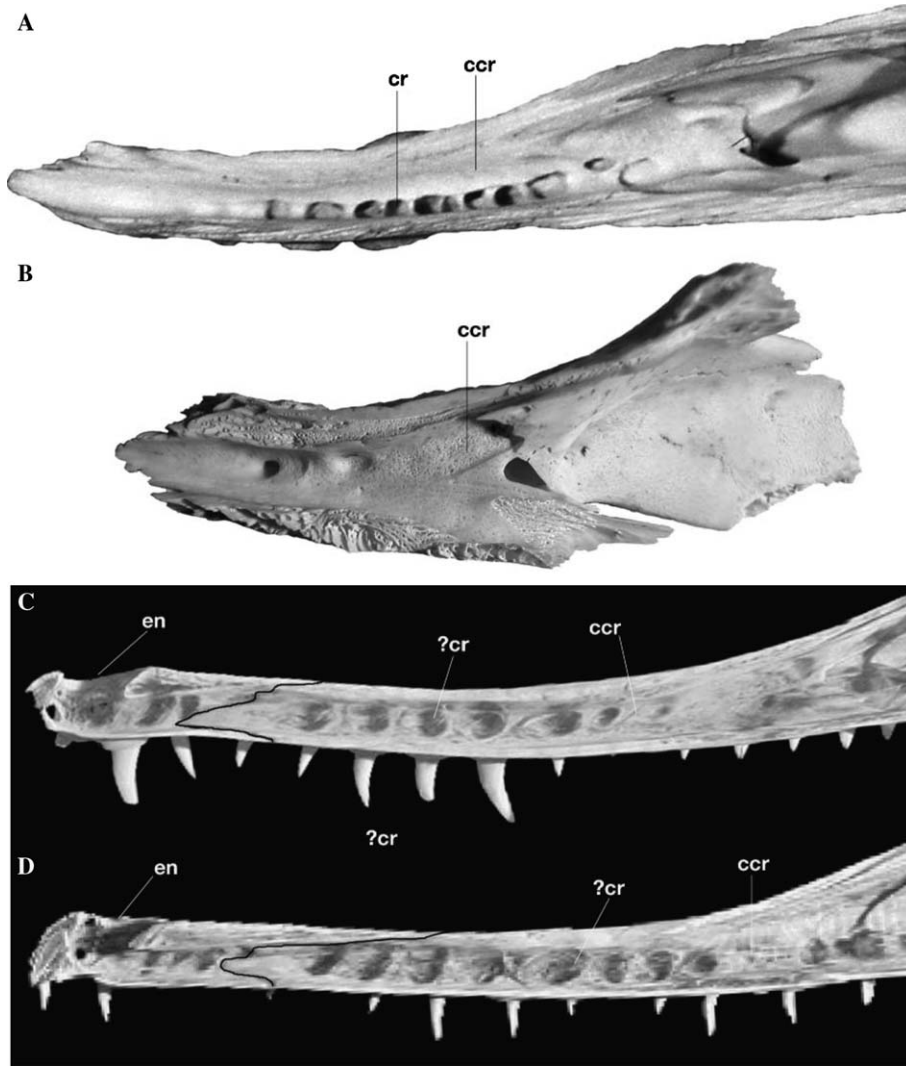


Fig. 7. Right maxillae, medial view, showing medial wall of caviconchal recess, (ccr); cr, caecal recess; and en, external naris. (A) UCMP 140795, *Crocodylus niloticus*. (B) UF34784, *Osteolaemus tetraspis*. (C) TMM m-3529, *Mecistops cataphractus*. (D) TMM m-6342, *Tomistoma schlegelii*.

4.5.2. Constrained morphological analysis

The constrained analysis increased tree length by less than one percent (from 452 to 448 steps), and differences are not significant based on a Wilcoxon signed rank test. *Mecistops* assumes a phylogenetic position outside other osteolaemines.

The new position of *Mecistops* produces a loss of resolution within *Crocodylus*. In the unconstrained analysis, *C. niloticus* is the sister taxon to a monophyletic New World clade; there is a monophyletic Indopacific assemblage within which *C. palustris* and *C. siamensis* branch near the base of the tree, and the position of one incompletely known fossil (*C. palaeindicus*) is unresolved. In the constrained results, the relationships of *C. niloticus*, *C. palustris*, and *C. siamensis* collapse. This partially reflects lability in *C. niloticus*—in some constrained optimal trees, *C. niloticus* is closer to *C. palustris*, and these two are sometimes closer to *C. siamensis*.

The cranial features diagnosing Osteolaeminae or subordinate nodes (Brochu, in review) are absent from *Mecistops*.

In fact, skeletal synapomorphies uniting *Mecistops* with *Osteolaemus*, or with any extinct osteolaemine, are absent, and the only unambiguous support comes from a horizontally-oriented anterior half to the axial neural spine, a feature with low CI. However, one morphological character that only ambiguously diagnoses *Crocodylus* in the unconstrained analysis—a nuchal shield with four central and two lateral osteoderms (Fig. 9)—becomes an unambiguous synapomorphy.

5. Discussion

Based on genetic diversity in the mitochondrial *ND6-cytb* and control region sequences, and for *C-mos* and *ODC* nuclear sequences, the earliest phylogenetic split within extant *Crocodylus* separates *M. cataphractus* from all the remaining species of *Crocodylus* (Avice and Walker, 1999). The genetic distances in Table 2 clearly indicate that *M. cataphractus* has achieved a level of divergence equal to or greater than that seen between the two genera, *Crocody-*

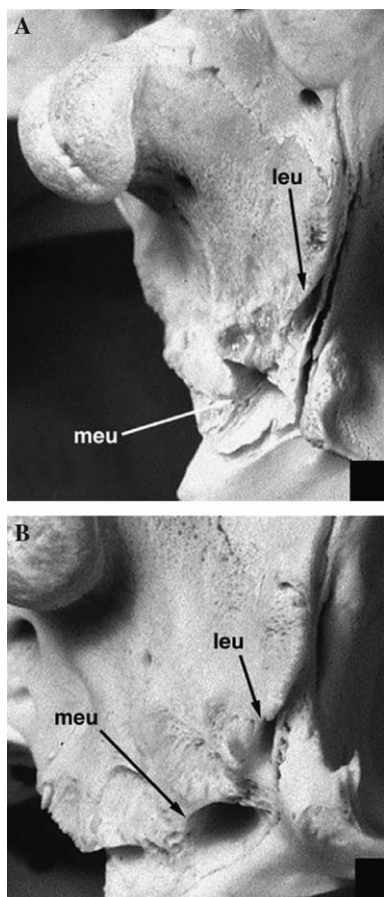


Fig. 8. Posteroventral portion of braincase, right ventrolateral view, for (A) TMM m-3529, *Mecistops cataphractus*, and (B) TMM m-1786, *Crocodylus niloticus*, showing relationship between lateral eustachian foramen (leu) and median eustachian foramen (meu).

lus and *Osteoleamus* (currently considered a valid monotypic genus).

Both nuclear markers used in this study are relatively novel in their phylogenetic application and provide some resolution at the intergeneric level. In all analyses performed, *M. cataphractus* is consistently outside a clade containing the remaining members of the genus *Crocodylus*. The inclusion of both mitochondrial and nuclear data refutes arguments that we are recovering only a “gene tree”

rather than actual evolutionary relationships, which is further supported by the inclusion of concatenated analyses of the data. One possible alternative explanation for relationships suggested by our data is long-branch attraction, and an examination of only mitochondrial distance values might lend credence to such a conclusion (White and Densmore, 2000). However, since divergence values for both nuclear datasets are much lower and certainly not anywhere close to saturation, long-branch attraction is most likely excluded as a viable explanation. We find it much more likely that *M. cataphractus* represents the sole surviving member of an ancient lineage endemic to the African continent. This idea is also supported by morphological evidence (Brochu, 2003).

Moving *Mecistops* closer to *Osteoleamus* has a minimal impact on our understanding of historical biogeography and the evolution of snout shape among crocodylids (Fig. 10). The clade including *Crocodylus* and Osteolaeminae is unambiguously of African origin regardless of where *Mecistops* is placed, and the highly derived slender snout of *Mecistops* arose independently of those of Tomistominae and *Euthecodon*, indicating at least three separate derivations (in addition to the two taxa with somewhat less derived snouts within *Crocodylus*) among crocodylids.

It would, however, have impact on crocodylian biostratigraphy and minimum divergence dates for extant genera. The oldest fossils referable to *M. cataphractus* are from the Late Miocene (Storrs, 2003; Tchernov, 1986). Of the extinct species thought to be closely related to *Mecistops*, one (“*Crocodylus*” *megarhinus*) lies outside the osteolaemine-*Crocodylus* clade and another (*C. nkodoensis* Pickford, 1994) is based on limited mandibular material that, though resembling corresponding parts of extant *M. cataphractus*, does not share discrete derived similarities with it. The oldest known crown *Crocodylus* (*C. palaeindicus*) predates the oldest *M. cataphractus*, so moving *Mecistops* closer to Osteolaeminae does not change the first appearance datum of *Crocodylus*; but the oldest osteolaemines (*Rimasuchus* and *Euthecodon*) date from the Early Miocene and extend the minimum divergence between *Mecistops* and *Crocodylus* from 12 to between 20 and 24 million years. This could be important to studies using crocodylid calibration points in molecular dating analyses (e.g., Brochu, 2004b).

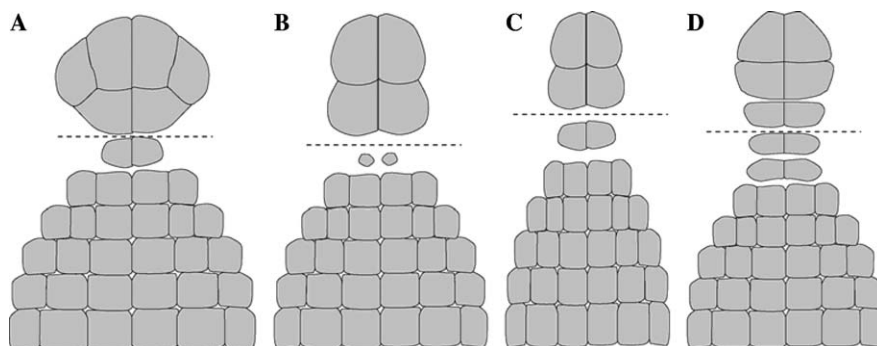


Fig. 9. Osteoderm patterns in crocodylians. Diagrams show nuchal shield (above dashed line) and anteriormost rows of dorsal shield (below dashed line) for (A) *Crocodylus niloticus*, (B) *Osteoleamus tetraspis*, (C) *Mecistops cataphractus*, and (D) *Tomistoma schlegelii*. Modified from Ross and Mayer (1983).

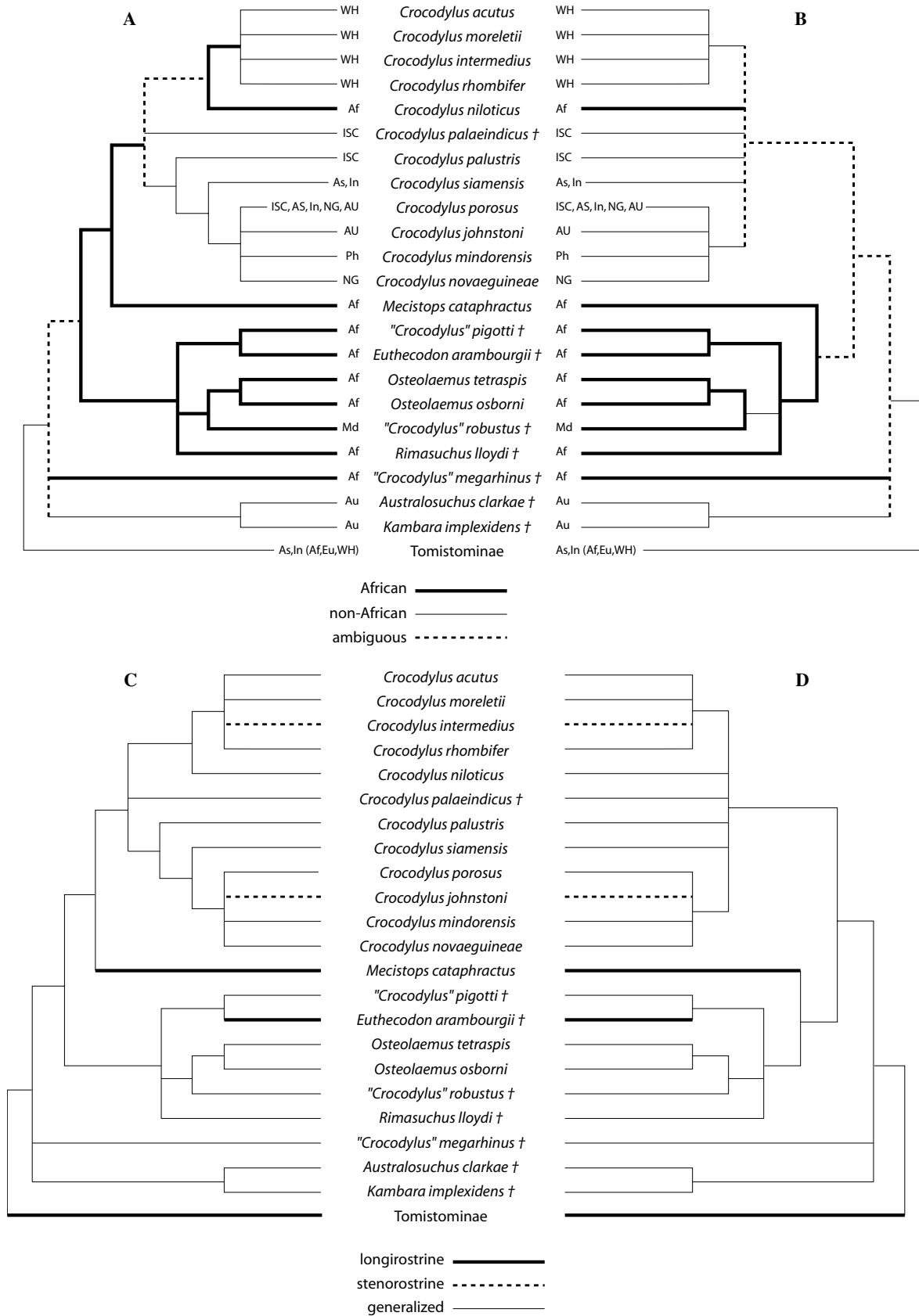


Fig. 10. Phylogenetic distribution of biogeographic occurrence (above) and snout shape (below) among crocodylids for the preferred morphological topology (A and C) and results constrained to draw *Mecistops* closer to *Osteolaemus* (B and D). For C and D, longirostrine refers to taxa in which the snout is both slender and elongate relative to overall skull size; stenorostrine taxa have relatively slender snouts lacking derived elongation.

Our conclusion that *Mecistops* is generically separate from *Crocodylus* mirrors that of Aoki (1976, 1992), but for very different reasons. Aoki argued that *Mecistops* forms a clade with *Gavialis* and *Tomistoma*, partially on the basis of similarities in the retroarticular process, including great relative length and what he described as a lateral rotation of the dorsal surface of the process itself. This lateral rotation appears to reflect an increased height of the process midline crest and a lateralward shift in its position rather than an actual rotation. Although he argued that these features are independent of snout shape, elongated retroarticular processes with higher midline crests are found in most longirostrine crocodyliforms (Brochu, pers. obs.). The broader medial convexity of the process in dorsal view, which was argued to indicate a closer relationship between *Mecistops* and *Tomistoma* (Endo et al., 2002), is common to most crocodylids.

The morphological dataset used here does not support these hypotheses. Shifting *Mecistops* to the base of Tomistominae (with *Gavialis* and its relatives phylogenetically outside a group containing other living crocodylians, which is the most parsimonious arrangement for morphological data) increases tree length by 11 steps, and moving *Mecistops* crownward closer to *Tomistoma* itself adds an additional eight steps. Forcing a clade including *Mecistops*, *Gavialis* and its extinct relatives (Gavialoidea), and Tomistominae increases tree length by at least 26 steps, and in this case *Mecistops* is outside the entire longirostrine clade, with tomistomines paraphyletic with respect to Gavialoidea. Maintaining a monophyletic Gavialoidea and making *Mecistops* the sister taxon to Tomistominae increases tree length to 39 steps longer than optimal. Adding characters to express retroarticular process similarities would not change these results significantly.

The concerted view of all datasets included herein as well as most other recent molecular and morphological analyses (Brochu, 1997; Densmore, 1983; Densmore and Owen, 1989; Gatesy et al., 2003; Gatesy et al., 2004; Schmitz et al., 2003; White and Densmore, 2000) supports that *M. cataphractus* is genetically distinct from the remaining *Crocodylus* species. While genetic distance values may be relatively low within our nuclear datasets, they strongly suggest that there is a real phylogenetic division within currently recognized *Crocodylus*. When genetic distance values are viewed in conjunction with phylogenetic relationships there is strong evidence for the exclusion of *M. cataphractus* from the remaining *Crocodylus*. Therefore, we are compelled to recommend the resurrection of the historic genus *Mecistops* (Gray 1844).

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Appendix A. Taxa used in morphological analysis

Tree topology for taxa not shown in Figs. 6 and 10 can be found in Brochu (2004a). Names in boldface are extant; remainder are extinct forms known only from fossils.

Outgroups: *Bernissartia fagesii*, *Hylaeochampsia vectiana*.

Gavialoidea: *Eothoracosaurus mississippiensis*, *Thoracosaurus neocesariensis*, *Thoracosaurus macrorhynchus*, *Eosuchus minor*, *Eosuchus lerichei*, *Eogavialis africanus*, *Argochampsia krebsi*, *Gryposuchus colombianus*, *Ikanogavialis gameroi*, *Gavialis lewisi*, ***Gavialis gangeticus***.

Stem brevirostrines: *Borealosuchus formidabilis*, *Borealosuchus wilsoni*, *Borealosuchus acutidentatus*, *Borealosuchus sternbergii*, *Pristichampsus vorax*.

Alligatoroidea: *Leidyosuchus canadensis*, *Diplocynodon darwini*, *Stangerochampsia mccabei*, *Brachychampsia montana*, ***Alligator mississippiensis***, ***Caiman yacare***, ***Paleosuchus trigonatus***.

Stem crocodyloids: *Brachyuranochampsia eversolei*, *Belgian crocodyloid*, *Crocodylus acer*, *Crocodylus affinis*, *Asiatosuchus grangeri*, *Asiatosuchus germanicus*, *Prodiplocynodon langi*.

Tomistomines: ***Tomistoma schlegelii***, *Tomistoma lusitanica*, *Paratomistoma courtii*, *Gavialosuchus americanus*, *Gavialosuchus eggenbergensis*, *Tomistoma cairensis*, *Kentisuchus spenceri*, *Dollosuchus dixonii*.

Mekosuchines: *Australosuchus clarkae*, *Kembara implexidens*.

Osteolaemines: ***Osteolaemus tetraspis***, ***Osteolaemus osborni***, *Rimasuchus lloidi*, “*Crocodylus*” *pigotti*, *Euthecodon arambourgii*, “*Crocodylus*” *robustus*.

Crocodylus: ***Crocodylus niloticus***, ***Crocodylus porosus***, ***Crocodylus rhombifer***, *Crocodylus palaeindicus*, ***Crocodylus acutus***, ***Crocodylus palustris***, ***Crocodylus siamensis***, ***Crocodylus intermedius***, ***Crocodylus johnstoni***, ***Crocodylus mindorensis***, ***Crocodylus novaeguineae***, ***Crocodylus moreletii***.

Other crocodylids: ***Mecistops cataphractus***, “*Crocodylus*” *megarhinus*.

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