AMER. ZOOL., 29:831–841 (1989)

Molecular Systematics of the Order Crocodilia[1]

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SYNOPSIS. Analyses of proteins, lipids and nucleic acids have been extremely useful for assessing the level of molecular divergence in the Crocodilia and for inferring the relationships of crocodilians to each other and to other vertebrates. A large body of traditional (morphological and paleontological), as well as recent non-traditional (cytogenetic and molecular) evidence concurs that birds are the closest living sister group to the Crocodilia. Relationships within the order have been much more difficult to resolve using traditional analyses due to the problems of convergence/parallelism and general morphological conservatism. Studies of protein divergence, while in agreement with traditional interpretations of affinities between the alligators and caimans, suggest that the true and false gharials are more closely related to each other than to other crocodilians and that the true crocodiles are all very close relatives that may have diverged recently. Preliminary analyses from an ongoing study of restriction endonuclease analysis of crocodilian mitochondrial and ribosomal DNAs corroborate both of these observations, suggesting that the molecular approach will be very valuable for resolving crocodilian phylogeny.

INTRODUCTION

The last two decades have witnessed a revival in crocodilian biology largely due to the classic studies on alligator metabolism by Roland Coulson, his colleagues and students. As the symposium can attest, this interest in crocodilians has not been restricted to physiological chemistry, but has included new insights into their behavior, development, ecology and even systematics and evolution. Regarding the latter two, relationships among crocodilians and their evolutionary history have traditionally been the exclusive realm of the comparative morphologist and vertebrate paleontologist. Whereas these disciplines continue to contribute much of the new data (especially those groups looking at innovative character sets; see Tarsitano *et al.*, 1989), a number of investigators have started to use non-traditional characters and novel approaches in attempts to resolve the natural affinities and evolutionary history of the living crocodilians. These range from analysis of coevolving crocodilian parasite lineages to "southern blot" and sequence data from mitochondrial and nuclear DNA. This paper will review the molecular aspects of crocodilian evolution, including biochemical and immunological studies of proteins as well as nucleic acid analyses, and relate the initial findings from a study of mitochondrial and ribosomal DNA evolution in crocodilians that is currently in progress.

REVIEW

How crocodilians are related to other vertebrates

Crocodilians are the sole living reptilian representatives of the subclass Archosauria. Protein sequence analysis of myoglobin (Dene *et al.*, 1980) and hemoglobin α and β chains (LeClercq *et al.*, 1981, 1982; Perutz *et al.*, 1981) as well as α-crystallin (DeJong *et al.*, 1985), immunological similarities of pancreatic polypeptides (Langslow *et al.*, 1973), DNA hybridization (Williams and Piatogorsky, 1979) and genome organization studies (Epplen *et al.*, 1979) are concordant with the most recent morphological studies (Rowe, 1986) in aligning crocodilians with birds and dinosaurs.

The Crocodilia were highly speciose during the Mesozoic Era and showed an

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[1] From the Symposium on *Biology of the Crocodilia* presented at the Annual Meeting of the American Society of Zoologists, 27–30 December 1987, at New Orleans, Louisiana.

incredible range in diversity, but are relictual today. Only 8 of the 124 described genera survive to the present and these are all members of the same suborder, Eusuchia. Twenty-one extant species (27 or 28 named forms) are currently recognized by most systematists. *Crocodylus* is by far the largest crocodilian genus, containing 11 living species.

Relationships among the extant Eusuchia

The Eusuchia probably arose during the late Mesozoic Era. Progenitors of the living genera may have diverged from other eusuchians as early as the Cretaceous. *Crocodylus* (the true crocodiles) probably represents the oldest extant genus and is known from at least the early Tertiary of Europe (Steel, 1973). One genus containing only long-snouted (longirostrine) species, *Tomistoma* (the false gharial), is represented in the Eocene of Asia (Steel, 1973), while the earliest representative of the other, *Gavialis* (the true gharial), occurs in the Miocene of South America and the Oligocene of India (Lull, 1944; Langston, 1965). The broad-snouted (brevirostrine) genus, *Alligator* (alligators), is known from the Miocene of North America (Malone, 1979), while the Neotropical genera *Caiman* (the true caimans) and *Paleosuchus* (the smooth-fronted caimans) first appear in the Pliocene (Steel, 1973). Two other extant genera, *Melanosuchus* (the black caiman) and *Osteolamus* (the dwarf African crocodiles), have not yet been found in the fossil record.

The natural affinities among living crocodilians have been determined primarily on the basis of comparative morphology. Figure 1 illustrates four different evolutionary trees all based upon traditional interpretations of crocodilian morphology. Whereas most authors have been consistent in aligning *Osteolamus* with *Crocodylus* and the caimans (*Caiman, Melanosuchus* and *Paleosuchus*) as the nearest sister taxa of *Alligator*, both the level and degree of relatedness are less definitive. Furthermore, there has been little or no agreement on the affinities of the two gharial genera (either to each other or to other crocodilians) using the traditional approach.

One major problem in resolving the systematics and evolution of the eusuchian crocodilians is their tendency towards overall morphological conservatism. This is especially true post-cranially, where there are few reliable characters that can be used for phylogenetic studies (Sill, 1968). Fortunately, there are numerous differences in head morphology and skull structure between different species, and most traditional assessments of crocodilian phylogeny are based upon analysis of these characters. This cranial variability strongly reflects variation in ontogeny (Steel, 1973) or habitat and diet (Iordansky, 1973). By example, there is an elongation of the snout and reduction of tooth size associated with the capture of fish, and some of the taxa having this condition undoubtedly share recent common ancestors. Unfortunately, such a lifestyle and its concomitant consequences on head shape have been very common throughout crocodilian history, presumably in widely divergent lineages. Even today there are caimans, crocodiles and gharials with elongated snouts and reduced teeth. Similar examples can be cited among brevirostrine forms which tend to have "generalist" diets, such as the alligators, most other caimans and even certain crocodiles. It is clear from these examples that similar adaptive strategies have led to convergent skull morphology and head shape in very different groups of crocodilians. Such convergence in character states that have long been considered phylogenetically important only complicates the interpretation of systematic relationships and evolution in crocodilians (Langston, 1973).

Intergeneric affinities among the extant Crocodilia

Several studies have employed analyses of non-traditional characters in attempts to resolve both intergeneric and interspecific affinities among the living Crocodilia. Cohen and Gans (1970) examined the chromosome morphology of all known species, and while their data were largely descriptive and hence not amenable for generating a branching sequence, the overall similarity in karyotypes is consis-

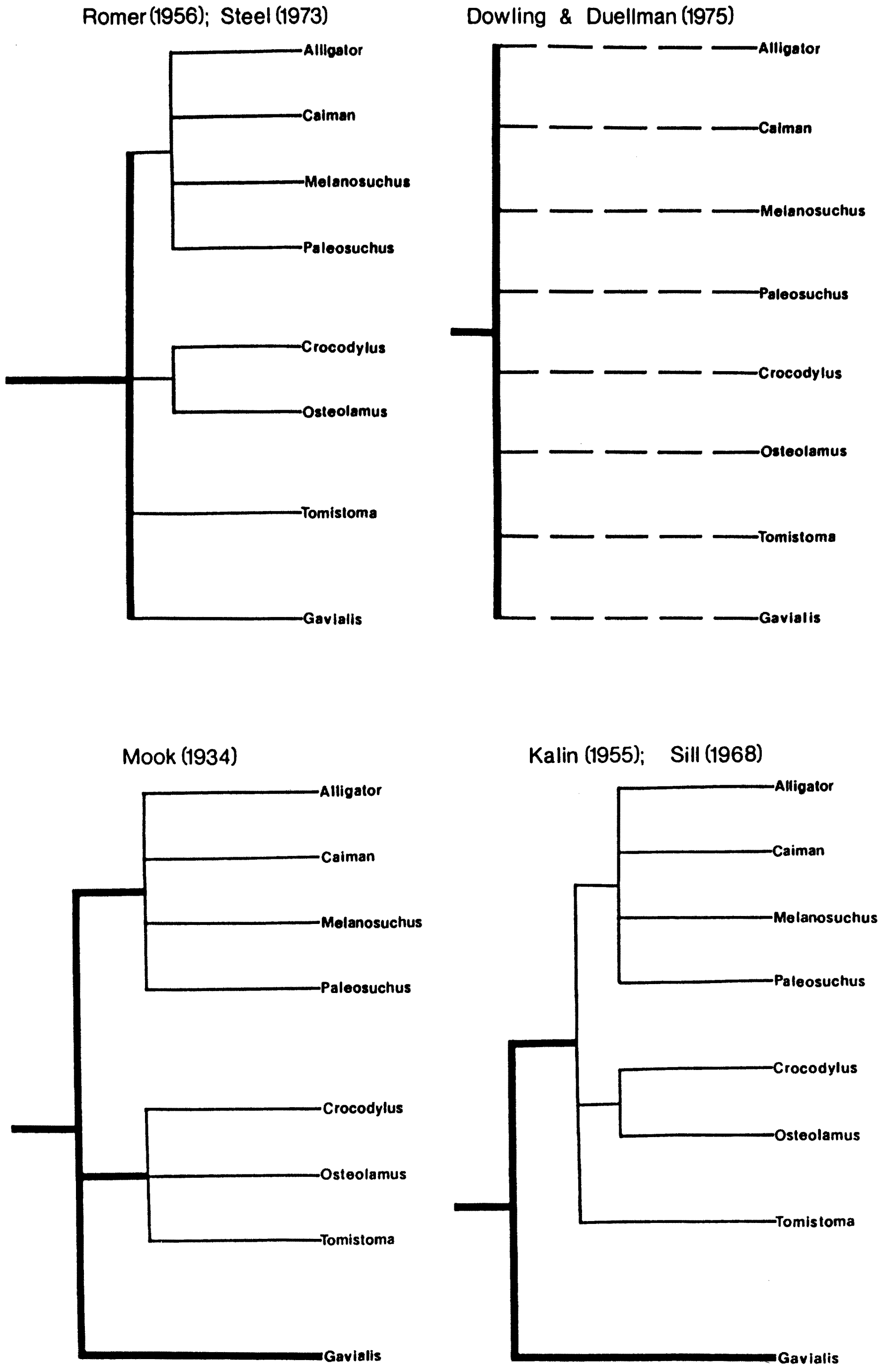

FIG. 1. Traditional interpretations of crocodilian systematics. The heavy lines indicate family level designation, the light lines either sub-family or genus. Three-family representations of Mook (1934) and Wermuth (1953), two-family representations of Kälin (1955) and Sill (1968), one-family representation of Romer (1956) and Steel (1973) and presumed one-family representation with no inferred relationships between genera of Dowling and Duellman (1975). Taken from Densmore (1983) with permission.

tent with a monophyletic origin of the living members of the order. However, *Alligator* and the caimans fell at opposite ends of a spectrum. King *et al.* (1986) recently completed a study of C-, G- and N-banding, as well as *in situ* hybridization of 18S and 26S ribosomal genes of chromosomes from three crocodilians, *Crocodylus porosus, C. johnsoni* and *Caiman crocodilus.* Their data suggest that extensive rearrangements have occurred that can be used to clearly differentiate the two crocodiles from the caiman, but that the overall organization of the euchromatic regions are conserved among these three taxa. Brooks (1979, 1981) studied digenean parasites that have apparently coevolved (and cospeciated) with crocodilian hosts to assess relatedness of taxa within the order. He found that the three caiman genera were distinct from one another and from *Alligator. Gavialis* is intermediate between *Alligator* and the two crocodile genera, although clearly sharing a more common digenean fauna with the latter. Brooks has not yet published data on the affinities of *Tomistoma.*

Studies by one of us employed biochemical and immunological analyses of proteins (Densmore, 1983; Dessauer and Densmore, 1983; Densmore and Dessauer, 1984) and more recently, restriction endonuclease digestion of mitochondrial and ribosomal DNAs isolated from crocodilian blood samples to assess relationships among the living Crocodilia. Figure 2 illustrates Densmore's (1983) findings from albumin immunodiffusion data, which were concordant with similar comparisons using transferrins. This technique involves raising antibodies against specific plasma proteins, either isolated biochemically in the case of albumins or identified using radioactive ^{59}Fe and reacting them with plasma (containing antigen) from different animals on plates of trefoil design. Plates were scored on the basis of presence or absence of the precipitin reaction and spur formation and intensity (see Goodman and Moore, 1971 for details).

These data suggest that the true (*Crocodylus*) and dwarf African (*Osteolamus*) crocodiles were closely related sister taxa. The alligators and caimans formed a loose assemblage of taxa, although *Alligator* was distinct from the three caiman genera, *Caiman, Melanosuchus* and *Paleosuchus.* Perhaps the most interesting finding was that the true (*Gavialis*) and false (*Tomistoma*) gharials were closely related sister taxa. The transferrin analyses were extremely important towards this end, as antisera raised against the transferrins of either *Gavialis* or *Tomistoma* did not recognize the transferrins from any other crocodilian genus. This finding remains intriguing, especially in light of recent work of Sam Tarsitano and his colleagues (1989) suggesting the uniqueness of *Gavialis* (see this volume). The immunological data are strengthened by similar findings using "hemoglobin fingerprints" (globins isolated from red blood cells, digested with trypsin and analyzed electrophoretically) and analysis of 21 structural gene loci from red blood cells using starch gel electrophoresis (Fig. 3a, b).

Interspecific relationships

Morphological differences (primarily in scalation or head shape) are sufficient to taxonomically discriminate species within genera (Brazaitis, 1973), but to date have been little or no help in assessing phylogeny at this level. Even the fossil record of crocodilians offers little aid in assessing interspecific relationships, both because of the problems of convergence mentioned above and because epidermal structures such as scales do not fossilize well. Furthermore, the very recent fossil record, which presumably contains remains of the most recent ancestors of the living species, is less than adequate (or poor) for many genera. For example, no direct ancestors of any of the 11 extant species of *Crocodylus* have ever been found in deposits older than the Pliocene (Steel, 1973; M. Hecht, personal communication). Under such circumstances, attempts to interpret intrageneric affinities are largely fruitless.

The non-traditional methods again appear best suited for addressing crocodilian intrageneric questions. Both Brooks' digenean analyses and my own biochemical and immunological work have been reasonably successful in discriminating among

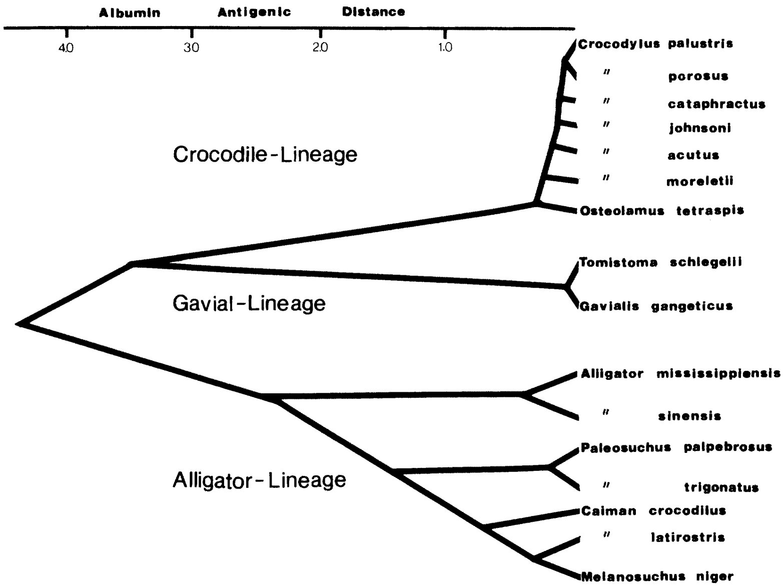

FIG. 2. UPGMA phenogram suggesting crocodilian relationships based on albumin immunodiffusion data. "Gavial" in this figure is equivalent to gharial elsewhere. Taken from Densmore (1983) with permission.

species within the several polytypic crocodilian genera. For example, within the genus *Caiman*, both of us clearly resolved *C. latirostris* from the *C. crocodilus* complex (see Figs. 2, 3a), although the biochemical data suggest that *Caiman* may be polyphyletic with respect to *Melanosuchus* (a finding recently corroborated by Mark Norell's unpublished analyses of new morphological characters). There is good overall agreement between the relationships suggested by the parasite, biochemical and immunological studies, with the glaring exception of our findings regarding the 11 species within *Crocodylus*. The results of Brooks' digenean analyses appear to resolve the relationships among the 11 species, while the protein phenotype data could only clearly differentiate *Crocodylus cataphractus* and *C. moreletii* from the other nine species, four of which (North America and Asia) could not be distinguished from one another using such characters (Fig. 3b). If we assume that the current circumtropical distribution of crocodiles is the result of a relatively ancient divergence, then Brooks' (1981) hypotheses of coevolution and cospeciation are probably correct. However, the current distribution of *Crocodylus* may be the result of a relatively recent (Pliocene) radiation that must have included at least one transoceanic migration. Most of the evidence is indirect: the lack of Pre-Pliocene lineages that can be traced directly to extant crocodiles, as well as the extremely low levels of protein divergence that were found among the 11 species of *Crocodylus.*

Although the protein similarity may be argued away as a slowdown in the rate of molecular evolution, no such slowdown appears to have taken place between the two species of alligator (see Fig. 3a), which have apparently been separated since the Miocene (Malone, 1979). Furthermore, all members of the genus *Crocodylus* appear to have a sub-lingual salt gland that may allow them to survive in seawater for extended periods (Taplin and Grigg, 1981). At least two species, *C. acutus* and *C. porosus,* may prefer brackish (and/or even salty) over freshwater habitats, with *C. porosus* actually having been sighted some 400 km offshore (Schmidt, 1957). If a recent radiation has occurred then the distribution of the digeneans and their parasite hosts may be due to a recent "sympatry" rather than a cospeciation event. One comparison that supports such a scenario concerns the affinities

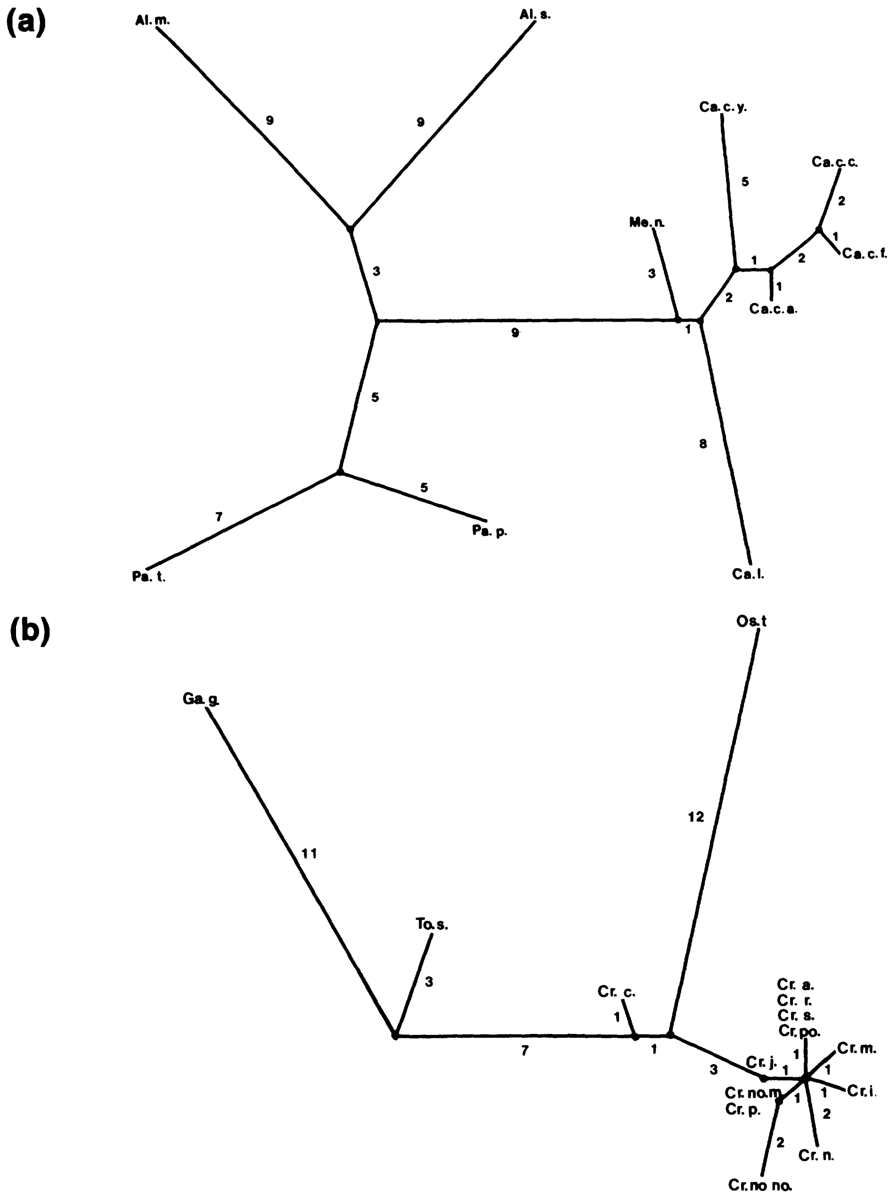

FIG. 3. Unrooted Wagner trees of allozyme phenotypes based on presence/absence of alleles. (a) relationships of taxa within the *Alligator* lineage. (b) relationships of taxa within the gharial and crocodile lineages. Abbreviations: Al.m., *Alligator mississippiensis*; Al.s., *A. sinensis*; Ca.c.a., *Caiman crocodilus apaporiensis*; Ca.c.c., *C. c. crocodilus*; Ca.c.f., *C. c. fuscus*; Ca.c.y., *C. c. yacare*; Ca.l., *C. latirostris*; Cr.a., *Crocodylus acutus*; Cr.c., *C. cataphractus*; Cr.i., *C. intermedius*; Cr.j., *C. johnsoni*; Cr.m., *C. moreletii*; Cr.n., *C. niloticus*; Cr.no.m., *C. novaeguineae mindorensis*; Cr.no.no., *C. n. novaeguineae*; Cr.p., *C. palustris*; Cr.po., *C. porosus*; Cr.r., *C. rhombifer*; Cr.s., *C. siamensis*; Ga.g., *Gavialis gangeticus*; Me.n., *Melanosuchus niger*; Os.t., *Osteolamus tetraspis*; Pa.p., *Paleosuchus palpebrosus*; Pa.t., *P. trigonatus*; To.s., *Tomistoma schlegelii.*

of *C. cataphractus* (the African slender-snouted crocodile). Brooks' data suggest that the species most closely related to *C. cataphractus* is *C. niloticus* (the Nile crocodile). The biochemical analyses showed *C. cataphractus* as the most distinct of all crocodiles (Fig. 3b), a finding shared by Cohen and Gans' (1970) analyses of crocodilian chromosomes.

This disagreement regarding the affinities of the 11 species of *Crocodylus* is interesting not only from a systematic aspect, but also because it may indicate that there may be drastic differences in the rate of molecular evolution among different crocodilian lineages and or convergence at the protein level. New studies employing different character sets must be completed to answer some of these difficult questions raised above. Analyses of other parasite fauna are being undertaken by Brooks and his co-workers (their paper this symposium, 1989) to test phylogenies based upon the digenean work.

We are currently comparing mitochondrial and ribosomal DNAs from critical crocodilian species to test two major phylogenetic hypotheses: 1) The two gharials are not sister taxa; and 2) The vicariance event(s) leading to the current distribution of the true crocodiles occurred further in the past than are suggested by the biochemical and immunological data.

Mitochondrial and ribosomal DNAs

Mitochondrial DNA (mtDNA) has rapidly become one of the most widely studied molecules for evolutionary biology. Vertebrate mtDNA is a closed-circular, double-stranded molecule that ranges in size from about 15,500 to over 27,000 base pairs. The mitochondrial genome is uniclonally inherited through the maternal parent, so that all mtDNA molecules within a single individual are essentially identical (although cases of substitutional and size heterogeneity are known; see Brown, 1985 for review). Gene order is highly conserved in all vertebrates examined to date and there are very few regions of non-coding or repetitive sequences (Brown, 1983, 1985). Despite this conservation, nucleotide sequence and high-resolution mapping studies suggest that mtDNA apparently evolves up to 5–10 times more rapidly than nuclear DNA (Brown *et al.*, 1979, 1982). This observation, now verified in many different vertebrate groups (see Brown, 1983, 1985, and Honeycutt and Wheeler, 1986) makes mtDNA very valuable for determining phylogenetic relationships among closely related taxa. Although mtDNA sequence analysis is not yet practical for most systematic studies, a large number of nucleotide substitutions can be detected by electrophoretically comparing mtDNAs which have been digested with different restriction endonucleases.

Ribosomal DNA is found in the eukaryotic nucleus and plays a structural role in the ribosome. Eukaryotic rDNA normally consists of three genes clustered together, designated 18S (S = Svedberg unit, a measure of the size and shape of the molecule in a density gradient), 5.8S and 28S. Additionally, a 5S gene is normally separated away from this cluster. Most metazoans typically have at least several hundred copies of rDNA genes. The 18S, 28S and 5S genes tend to be highly conserved, while the internally transcribed spacers that separate them are more labile (see Gerbi, 1985, and Appels and Honeycutt, 1986, for recent reviews on structure and evolution). By isolating and cloning specific regions of the rDNA, hybridization probes can be produced which can be used to detect either slowly or rapidly evolving sequences.

Restriction endonucleases are bacterial enzymes which recognize specific four, five or six base sequences of double-stranded DNA. By digesting mtDNA or rDNAs from different species with the same restriction enzyme and analyzing these digested samples on agarose or polyacrylamide gels, one can compare the sizes of fragments produced during the digestion. In most cases, the more closely related the species, the more similar are their restriction patterns.

METHODS AND MATERIALS FOR DNA ANALYSES

mtDNA samples used as probes were isolated from freshly dissected or quick-frozen crocodilian heart, liver and/or kidney using preparative ultracentrifugation according to the protocols of Wright *et al.* (1983) and Densmore *et al.* (1985). Either mtDNAs prepared as above from the American alligator (*Alligator mississippiensis*), 18S or 28S rDNAs (from mouse [*Mus musculus*], kindly provided by Drs. S. Davis, D. Hillis and N. Arnheim) were end-labelled and used as hybridization probes following "random priming" (Feinberg and Vogelstein, 1983).

Since most crocodilians are endangered

or protected, whole blood, collected according to Gorzula *et al.* (1976), was often the only tissue available for mitochondrial or ribosomal DNAs. Total DNAs (including both mitochondrial and nuclear fractions) from the Chinese alligator (*Alligator sinensis*), the African slender-snouted crocodile (*Crocodylus cataphractus*), the Orinoco crocodile (*C. intermedius*), Johnson's crocodile (*C. johnsoni*), Morelet's crocodile (*C. moreletii*), the Cuban crocodile (*C. rhombifer*), the Siamese crocodile (*C. siamensis*), the Indian gharial (*Gavialis gangeticus*) and the false gharial (*Tomistoma schlegelii*) were prepared by adapting methods from Maniatis *et al.* (1982). Following digestion with one or more restriction enzymes (see below) and electrophoresis, the mtDNA and ribosomal DNA fragments were visualized using "Southern blot" transfer followed by hybridization with the probe DNAs (Southern, 1975) and autoradiography (Brown, 1980).

RESULTS OF DNA ANALYSES

Total DNAs from four crocodilian species, *Alligator sinensis, Gavialis gangeticus, Tomistoma schlegelii* and *Crocodylus rhombifer,* were digested with eight restriction endonucleases that each recognize different six-base sequences (*Ava*I, *Bam*HI, *Eco*RI, *Hind*III, *Mlu*I, *Pst*I, *Stu*I and *Xho*I).

Following hybridization with *Alligator mississippiensis* probe mtDNA, restriction patterns of the four representative mtDNAs were compared. The length of the crocodilian mtDNAs was uniform, averaging about 17.4 kilobases (kb). Few restriction fragments were shared among the four mtDNAs. The two gharials shared the largest number of fragments; calculation of Upholt's (1977) P value produced estimates of sequence divergence that ranged from 7% between the two gharials to 12% between the true gharial and Chinese alligator. The sequence divergence estimates were used to generate a phenogram for the four genera (Fig. 4).

Mitochondrial DNAs from six representative species of *Crocodylus* (including *C. cataphractus, C. intermedius, C. johnsoni, C. moreletii, C. rhombifer, C. siamensis*) were also compared using the alligator mtDNA probe. The restriction enzyme patterns of the few enzymes used to date suggest that the mtDNAs of all of these species of *Crocodylus* are very similar to one another. In one case, there is no apparent difference in the *Hind*III fragment patterns from *C. siamensis* and *C. rhombifer* mtDNAs.

DISCUSSION

The mtDNA data presented here, while admittedly preliminary, corroborate the results of the biochemical and immunological studies discussed earlier (Densmore, 1983; Densmore and Dessauer, 1984). The alignment of the true and false gharials is especially interesting (Fig. 4). While it is clear that these two genera are far from identical, there are enough corroborative data sets to suggest that these taxa may be more closely related to each other than to any other living crocodilians. Concomitantly, it must be accepted either that the living Crocodilia are a monophyletic assemblage (Densmore and Dessauer, 1984), or that the entire order is polyphyletic. The similar fragment patterns from mtDNAs of true crocodiles suggest that they are not only closely related, but also may be recently derived (Densmore, 1983). However, it is clear that considerably more molecular data must be accumulated before any definitive statements can be made about relationships among the 11 different species of *Crocodylus.*

The cleavage mapping studies currently in progress should permit verification or refutation of hypotheses suggested by these preliminary data. By mapping each restriction site relative to other sites, one is able to ascertain whether restriction fragments of two mtDNA samples that comigrate are homologous. Restriction site data (as opposed to fragment data) are much more amenable to phylogenetic analysis.

Hybridization with the mouse ribosomal DNA probes to these same membranes is also contributing valuable information. Once the mtDNA analyses have been completed, the membranes are stripped in alkali and then rehybridized with one of the rDNA probes. Repeating this process with the other rDNA probe allows comparison of three different DNA markers (*i.e.*, mtDNA, 18S rDNA and 28S rDNA) from a single set of restriction digests. Due to

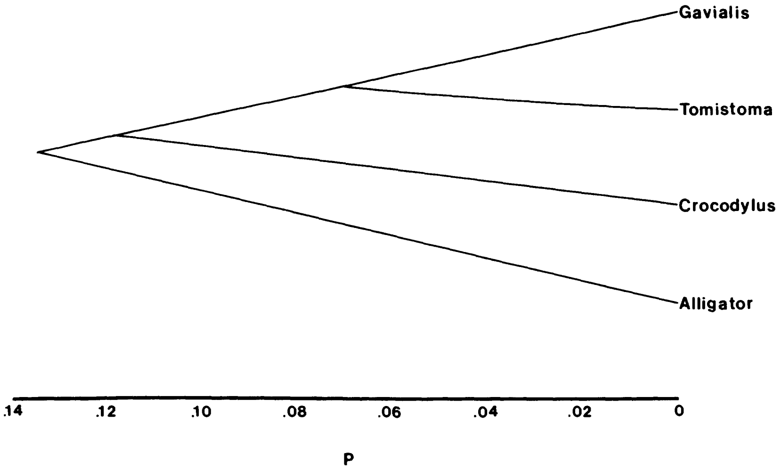

FIG. 4. UPGMA phenogram of Upholt's (1977) divergence measure (P) for mitochondrial DNA fragments from four crocodilian genera produced by digestions with eight restriction enzymes.

the conserved nature of the coding regions of the genes, restriction patterns of rDNA are typically less variable than mtDNA. So far at least two enzymes (*Ava*I and *Pst*I) show sufficient variation to be used for phylogenetic analysis. Increasing the number of restriction enzymes and mapping the restriction sites (see Hillis and Davis, 1986) should provide data suitable for testing hypotheses of crocodilian relationships.

Regardless of how the molecular results turn out, studies of the Crocodilia offer the very rare opportunity to examine variation at several taxonomic levels in an entire vertebrate order while comparing fewer than thirty named forms. Are we looking at a slowdown in molecular as compared to morphological evolution in the crocodiles and gharials? Are the living crocodiles as recently derived as all of the molecular data continue to suggest? Our studies and the continued work of Dan Brooks, Max Hecht, Mark Norell, Sam Tarsitano and their colleages will eventually resolve these and other interesting aspects of crocodilian systematics and evolution.

ACKNOWLEDGMENTS

We wish to thank the following institutions for their contributions of crocodilian blood or other tissue for these studies: the Atlanta Zoo, the Bronx Zoo, the Cincinnati Zoo, the Dade County (Miami) Zoo, the Houston Zoo, the Forth Worth Zoo, the LSU School of Medicine Frozen Tissue Collection, the National Zoological Park (Washington, D.C.), the Philadelphia Zoo, the Reptile Breeding Foundation (Picton, Ontario), the Rockefeller Wildlife Refuge (Grand Chenier, Louisiana), and the St. Augustine (Florida) Alligator Farm. In addition, without the help of these curators, scientists, wildlife biologists and friends, this work would have been impossible: R. Atkinson, J. Behler, J. Black, M. Bond, J. K. Bowler, P. Brazaitis, W. Brown, T. Coulson, M. Davenport, H. Dessauer, T. Dowling, F. and K. Earnest, C. Gans, M. Goodman, M. Hecht, T. Hernandez, D. Jardine, T. Joanen, V. Lance, D. Marcellini, C. Moritz, J. Murphy, H. Quinn, S. Rogers, M. Wise, and B. Ziegler. Several individuals read and commented on earlier versions of this manuscript including: M. Hamilton, A. Knight, C. Porter, O. Ryder and R. Van Den Bussche. W. Rains photographed the figures. This work has been supported in part by the National Science Foundation (DEB-7920663 and BSR 8607420) and by Texas Tech University. Finally, this publication is dedicated to Professor Roland Coulson, a leader in the field of crocodilian biology.

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