



Molecular phylogenetic analyses of genus *Crocodylus* (Eusuchia, Crocodylia, Crocodylidae) and the taxonomic position of *Crocodylus porosus*

P.R. Meganathan^a, Bhawna Dubey^a, Mark A. Batzer^b, David A. Ray^{b,1}, Ikramul Haque^{a,*}

^a National DNA Analysis Centre, Central Forensic Science Laboratory, 30, Gorachand Road, Park Circus, Kolkata 700 014, West Bengal, India

^b Department of Biological Sciences, Louisiana State University, 202 Life Sciences Building, Baton Rouge, LA 70803, USA

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ABSTRACT

The genus *Crocodylus* consists of 11 species including the largest living reptile, *Crocodylus porosus*. The current understanding of the intrageneric relationships between the members of the genus *Crocodylus* is sparse. Even though members of this genus have been included in many phylogenetic analyses, different molecular approaches have resulted in incongruent trees leaving the phylogenetic relationships among the members of *Crocodylus* unresolved inclusive of the placement of *C. porosus*. In this study, the complete mitochondrial genome sequences along with the partial mitochondrial gene sequences and a nuclear gene, C-mos were utilized to infer the intrageneric relationships among *Crocodylus* species with a special emphasis on the phylogenetic position of *C. porosus*. Four different phylogenetic methods, Neighbour Joining, Maximum Parsimony, Maximum Likelihood and Bayesian inference, were utilized to reconstruct the crocodylian phylogeny. The uncorrected pairwise distances computed in the study, show close proximity of *C. porosus* to *C. siamensis* and the tree topologies thus obtained, also consistently substantiated this relationship with a high statistical support. In addition, the relationship between *C. acutus* and *C. intermedius* was retained in all the analyses. The results of the current phylogenetic study support the well established intergeneric crocodylian phylogenetic relationships. Thus, this study proposes the sister relationship between *C. porosus* and *C. siamensis* and also suggests the close relationship of *C. acutus* to *C. intermedius* within the genus *Crocodylus*.

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1. Introduction

Crocodylia, a small order within the class Reptilia comprises 23 species belonging to eight genera (King and Burke, 1989), among which *Crocodylus* is the largest, represented by 11 species. Formerly, the genus *Crocodylus* was known to consist of 12 species including *Crocodylus cataphractus* but recent studies have provided consistent evidence for this species as a non-*Crocodylus* member (Brochu, 2000; McAilley et al., 2006) and thus the name, '*Mecistops cataphractus*' was resurrected. The genus *Crocodylus* has been included in many phylogenetic analyses, which have established the basic structure of the crocodylian phylogeny (Gatesy et al., 2003, 2004; Harshman et al., 2003; Janke et al., 2005; McAilley et al., 2006; Roos et al., 2007). These studies mostly aimed to resolve the interfamilial and intergeneric problems with few of them focusing on the intrageneric relationships of *Crocodylus*. However, the relationships between species within *Crocodylus* remain poorly understood.

The studies based on the morphological features, supported the monophyly of genus *Crocodylus* and also illustrated the presence of two crocodylian lineages i.e., the New World and the Indopacific assemblage (Brochu, 2000). The New World assemblage consists of *Crocodylus acutus*, *C. rhombifer*, *C. intermedius* and *C. moreletii*. Whereas, the Indopacific crocodylian lineage comprises of *C. novaeguineae*, *C. mindorensis*, *C. siamensis*, *C. porosus* and *C. johnstoni*. Morphological examinations have also suggested a polytomy between *Crocodylus niloticus*, the New World crocodylian and the Indopacific assemblage (Brochu, 2000). In addition these analyses also supported the close relationship of *C. palustris* to the Indopacific clade. Although some molecular studies supported the monophyly of *Crocodylus*, different molecular approaches have resulted in incongruent trees (Densmore, 1983; Densmore and Dessauer, 1984; Densmore and Owen, 1989; Densmore and White, 1991; Hass et al., 1992; Poe, 1996; McAilley et al., 2006; Li et al., 2007; Gatesy and Amato, 2008; Willis, 2009). Moreover, the New World and the Indopacific lineages as observed in the morphological examinations were not supported by some molecular studies (Densmore and Owen, 1989; Poe, 1996). However, the sister relationship between *C. novaeguineae* and *C. mindorensis* and the relationship between *C. acutus* and *C. intermedius* within *Crocodylus* has been recovered in many phylogenetic studies (Densmore and

* Corresponding author. Fax: +91 33 2284 9442.

E-mail address: haque_cfslk@yahoo.co.in (I. Haque).

¹ Present address: Department of Biochemistry and Molecular Biology, Mississippi State University, Starkville, MS 39762, USA.

White, 1991; Poe, 1996; White and Densmore, 2000; Ray, 2002; Gratten, 2003; McAliley et al., 2006; Oaks, 2007; Gatesy and Amato, 2008). The phylogenetic placement of the remaining species within genus *Crocodylus* has remained unclear and has not garnered much attention.

One such species having ambiguous phylogenetic position is the saltwater crocodile, *C. porosus*. This species is one of the largest living crocodylians having estuarine as well as fresh water as their main habitats and a wider geographical distribution in the Indopacific region than any other crocodylian species. The remaining species of the Indopacific clade are found in fresh water/marsh environment and rarely in brackish water (Martin, 2008). These interesting ecological features led us to examine the phylogenetic position of *C. porosus* within the genus *Crocodylus*. *C. porosus* has not been included in many phylogenetic analyses and those including this species could not provide a consistent placement for *C. porosus*. Previous molecular studies described its close association with *C. palustris* (Densmore and Owen, 1989; Poe, 1996; Gatesy and Amato, 2008; Willis, 2009), whereas some studies have combined *C. porosus* within the monophyletic clade of other Indopacific crocodylians excluding *C. siamensis* and *C. palustris* (Densmore, 1983; Densmore and Owen, 1989; Brochu, 2000). Nevertheless, these relationships could not gain good statistical support. McAliley et al. (2006) have shown a sister relationship of *C. porosus* with *C. siamensis* but a conclusive placement for this species was not emphasized. Hence, no consensus could be established regarding the phylogenetic position of *C. porosus*. This implies the need for further molecular studies to provide a better understanding of the relationships among *Crocodylus* species and to establish the phylogenetic position of *C. porosus*.

Herein, the crocodylian phylogeny is reconstructed using (i) complete mitochondrial genome (mt-genome), (ii) partial mtDNA gene sequences: 12S rRNA, NADH subunit 4 (ND4), NADH subunit 6 (ND6), cytochrome *b* (*cyt b*), tRNA^{glu}, control region, and (iii) a nuclear proto-oncogene *C-mos* (*C-mos*). The complete mt-genome data was included for the analyses because the increased length of sequence data increases the probability of obtaining correct tree topology (Rosenberg and Kumar, 2001; Wortley et al., 2005) and this phenomena was evident from the recent studies that have elucidated deeper level relationships in insects, bears and avian species using whole mt-genome (Cameron et al., 2007; Yu et al., 2007; Pratt et al., 2009). Moreover, Gatesy and Amato (2008) has emphasized on the need for whole mitochondrial genome analyses to resolve the relationships within Crocodylia. However, at present only 15 complete mt-genome sequences, including the mt-genome generated here, are available of the 23 existing crocodylian species. Therefore this study also analyses the partial 12S rRNA, ND4, ND6, *cyt b*, tRNA^{glu} and control gene sequences separately to support the mt-genome phylogeny. Furthermore, these sequences are available in public databases for species which are not found in India and that could be appended effectively to overcome the problems arising due to taxon sampling. Although the mt-genome data are known to infer the phylogenetic relationships accurately, the use of a nuclear gene to support the topology obtained from mt-genome has been emphasized (Springer et al., 2001; Stepan et al., 2005). Therefore, this study includes a nuclear gene *C-mos* which has already been proved useful in crocodylian phylogenetics (McAliley et al., 2006; Gatesy and Amato, 2008).

2. Materials and methods

2.1. Sample collection and DNA extraction

The authenticated biological samples were obtained from Madras Crocodile Bank Trust (MCBT), Centre for Herpetology,

Mamallapuram, Tamilnadu, India, National Chambal Sanctuary Project, Agra, Uttar Pradesh, India and Nehru Zoological Park, Hyderabad, Andhra Pradesh, India. Whole blood samples from *C. palustris*, *C. porosus*, *C. siamensis*, *C. johnstoni*, *C. niloticus*, *Caiman crocodylus* and *Gavialis gangeticus* as well as tissue samples of *G. gangeticus* were included in the present study. Genomic DNA extraction from blood samples was carried out using standard Phenol:Chloroform procedure (Sambrook and Russel, 2001) followed by purification with Microcon 100 centrifugal filter column (Millipore). DNA extraction from tissue samples was performed using DNeasy tissue kit (QIAGEN, GmbH, Germany) as per the manufacturer's guidelines.

2.2. Data sampling

The whole mt-genome for *C. palustris* and *C. johnstoni* were generated in this study using our indigenous primers (Meganathan, unpublished). The complete mt-genome of *C. moreletii* was obtained from the Laboratory of Computational Genomics, Louisiana State University (Ray, unpublished). The partial gene sequences of 12S rRNA, ND4, ND6, and control region sequences were amplified and sequenced for *C. porosus*, *C. niloticus*, *C. palustris*, *C. johnstoni*, *G. gangeticus* and *C. crocodylus*. Whereas, the ND4 and control region data for *C. novaeguineae* and *C. mindorensis* were generously provided by Dr. Gratten (2003). The tRNA^{glu}-*cyt b* gene sequences of *C. porosus*, *C. niloticus*, *C. palustris*, *C. johnstoni*, *G. gangeticus* and *C. crocodylus*, generated in our previous study (Meganathan et al., 2009a,b) were included in the analyses. The partial *C-mos* gene sequences of *C. palustris*, *C. porosus*, *C. siamensis*, *C. johnstoni*, *G. gangeticus* and *C. crocodylus* was amplified and sequenced using the primers *C-mos*-F: 5' ATA GTT GCT GTG AAG CAG GT 3' and *C-mos*-R: 5' GCT CAG TGA TGA ACA CAT TG 3'. The available complete mt-genomic sequences, partial mtDNA gene and the sequences of *C-mos* gene for the remaining crocodiles were retrieved from GenBank.

2.3. Data analyses

The whole mt-genome sequences of *C. palustris*, *C. johnstoni* and *C. moreletii* were aligned with the other crocodylian mt-genome sequences available in GenBank (Table 1) using MEGA 3.1 software (Kumar et al., 2004). The non-protein coding genes/regions as well as the start and stop codons of protein coding genes in the mt-genome were identified and removed. The partial gene sequences obtained were checked using BLAST (Altschul et al., 1990) to search for PCR contamination and artifacts. These sequences were aligned with the whole mitochondrial genome sequences of crocodylian species using MEGA 3.1 and edited by Bioedit software (Hall, 1999). The partial *C-mos* gene sequences was aligned with the other available partial *C-mos* sequence of crocodiles and the unaligned 5' and 3' segments were eliminated. On the basis of the

Table 1

List of complete mitochondrial genome sequences of crocodile species and their accession numbers as retrieved from NCBI database.

Species name	Accession number	References
<i>Alligator mississippiensis</i>	Y13113	Janke and Arnason (1997)
<i>Alligator sinensis</i>	AF511507	Wu et al. (2003)
<i>Paleosuchus palpebrosus</i>	AM493870	Roos et al. (2007)
<i>Paleosuchus tetraspis</i>	AM493869	Roos et al. (2007)
<i>Caiman crocodylus</i>	AJ404872	Janke et al. (2001)
<i>Gavialis gangeticus</i>	AJ810454	Janke et al. (2005)
<i>Tomistoma schlegelii</i>	AJ810455	Janke et al. (2005)
<i>Osteolaemus tetraspis</i>	AM493868	Roos et al. (2007)
<i>Mecistops cataphractus</i>	NC010639	Unpublished
<i>Crocodylus niloticus</i>	AJ810452	Janke et al. (2005)
<i>Crocodylus siamensis</i>	DQ353946	Ji et al. (2008)
<i>Crocodylus porosus</i>	AJ810453	Janke et al. (2005)

availability of sequence data from other *Crocodylus* species, the sequences were concatenated and grouped into six data sets: (a) whole mt-genome data, (b) ND6–cyt *b*–tRNA^{glu}, (c) ND4–control region, (d) ND6–cyt *b*–tRNA^{glu}–ND4–control region (e) 12S rRNA–cyt *b* and (f) C-mos dataset and each data set were analyzed separately. The ambiguous positions were removed manually but the gaps were not removed as the gaps are known to contain valuable information for phylogenetic analyses (Simmons and Ochoterena, 2000).

Four types of phylogenetic analyses were carried out: Neighbour Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian analysis. In all analyses *Alligator mississippiensis* was assigned as the out group. The nucleotide substitution models based on Akaike Information Criteria (AIC) are known to perform accurately (Posada and Buckley, 2004), therefore the best fit models for ML and Bayesian analyses for each gene were selected separately under AIC using jModelTest (Guindon and Gascuel, 2003; Posada, 2008). The uncorrected pairwise distances (*p*-distances) were calculated using MEGA 3.1 and genetic distances were calculated under F81 nucleotide substitution model (Felsenstein, 1981) with 1000 bootstrap replicates using dnadist program as implemented in Phylip 3.68 software package (Felsenstein, 1993). The ML distances were calculated in Tree-Puzzle 5.2 software (TP) (Schmidt et al., 2002) under three nucleotide substitution models, GTR (Lanave et al., 1984; Rodriguez et al., 1990), TN (Tamura and Nei, 1993) and HKY (Hasegawa et al., 1985). All the pairwise distances obtained were used to construct NJ tree in Phylip. The trees constructed under F81 model distances were summarized to a strict consensus tree using CONSENSE program in Phylip package. For MP analyses, two methods: heuristic search (Fitch, 1971) as implemented in dnaps and a branch and bound search (Hendy and Penny, 1982) available in dnappenny program, were used to find out the most parsimonious tree with 1000 bootstrap replicates. The majority rule consensus tree was constructed separately for each analysis in Phylip.

For ML and Bayesian analyses the genes were partitioned within the dataset and analyzed as partitioned data with mixed models. The ML partition analyses were carried out in PAML 4.3 software (Yang, 2007). In order to check the consistency of topology, ML analyses were also performed using PAUP 4.0 beta (Swofford, 1998) and PHYML (Guindon et al., 2005). Quartet puzzling trees were constructed for all datasets in TP (quartet puzzling algorithm) using three substitution models, GTR, TN and HKY. The robustness of ML tree was analyzed by evaluating the log-likelihood values (log *L*) with bootstrap (1000 replications) support. The Bayesian analyses was performed in MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001). To evaluate the parameters used, a metropolis-coupled MCMC was run with six incremental chains. A starting chain was chosen at random, 1.0×10^7 generations were run with sampling at every 100th generation. The first 10% trees were discarded and the resulting trees were used to generate a majority consensus tree with posterior probabilities. To select the best tree topology, the Shimodaira–Hasegawa (SH) (Shimodaira and Hasegawa, 1999) test was performed using TP software.

3. Results

This study utilized six data sets including the whole mt-genome to extensively analyze the relationships between *Crocodylus* species and to establish the phylogenetic position for *C. porosus* within *Crocodylus*.

3.1. Whole mt-genome analyses

The complete mt-genome sequences of *C. palustris* (16,852 bp), *C. johnstoni* (16,851 bp) and *C. moreletii* (16,827 bp) were aligned with other complete mt-genome sequences of crocodiles (Table 1). After removing the non-protein coding sequences and start and stop codons of protein coding genes 11,460 bp sequences were utilized for the analyses. This protein coding whole mt-genome dataset reveals 5213 constant, 6230 variable and 4955 parsimony informative sites. The uncorrected pairwise distance between *C. porosus* and *C. siamensis* was 1.57% which was found to be the lowest (Table 2). The highest distance was obtained for *C. siamensis* to *C. johnstoni* (10.9%).

All the analyses based on this dataset produced identical trees and supported the established interfamilial crocodylian relationships. The results agree to the monophyly of *Crocodylus* and produced two separate lineages, New World and Indo Pacific groups. This dataset strongly supports the close relationship between *C. porosus* and *C. siamensis* within the genus *Crocodylus* (bootstrap = 100; posterior probability = 1.00). The sister relationship between *G. gangeticus* and *T. schlegelii* was retained with strong nodal support and the non-*Crocodylus* status of *M. cataphractus* was supported by this analyses. The result for this data set is shown in Fig. 1.

3.2. ND6–tRNA^{glu}–cyt *b* data analyses

Analyses of 300 bp of sequence spanning the ND6, tRNA^{glu} and cyt *b* genes yielded 108 conserved, 188 variable and 131 parsimony informative sites. The alignment shows three bp deletions (nlt position 154–156) in the members of family Alligatoridae. A two bp insertion was present only in *C. intermedius* at the position 160 and 161 thus leading to a total deletion of five bp (nlt position 159–163) in *C. mindorensis* as well as *C. novaeguineae*. The genetic distance was very low between *C. acutus* and *C. intermedius* (0.7%) and the highest distance, 10.5% was noticed between *C. johnstoni* and *C. intermedius*.

This data set including all eleven *Crocodylus* species unambiguously retained the accepted crocodylian phylogeny, including the monophyly of *Crocodylus* and also adequately resolved certain relationships within the genus *Crocodylus*. All the analyses resulted in similar tree topologies, with small variations and the result is shown in Fig. 2. The analyses except Bayesian inference show the separate clade for New World crocodylians but did not

Table 2

Uncorrected pairwise distance values for six data sets.

Data set → Relationship between ↓	Whole mt-genome (in %)	ND6–cyt <i>b</i> –tRNA ^{glu} (in %)	ND4–control (in %)	ND6–cyt <i>b</i> –tRNA ^{glu} – ND4–control (in %)	12S rRNA– cyt <i>b</i> (in %)	C-mos (in %)
<i>C. porosus</i> to <i>C. siamensis</i>	1.57	1.1	2.4	2.1	1.6	0.00
<i>C. porosus</i> to <i>C. palustris</i>	9.27	7.8	6.7	7.0	7.9	0.27
<i>C. porosus</i> to <i>C. johnsoni</i>	10.69	9.7	9.1	9.2	8.9	0.81
<i>C. siamensis</i> to <i>C. palustris</i>	9.73	8.2	7.0	7.3	9.2	0.27
<i>C. siamensis</i> to <i>C. johnsoni</i>	10.99	10.1	10.1	10.05	10.2	0.8
<i>C. acutus</i> to <i>C. intermedius</i>	NA	0.7	NA	NA	1.3	0.00
<i>Mecistops</i> to <i>Crocodylus</i>	16.88	14.91	13.4	13.8	12.7	1.2
<i>Mecistops</i> to <i>Osteolaemus</i>	15.98	16.4	14.4	14.7	11.8	NA

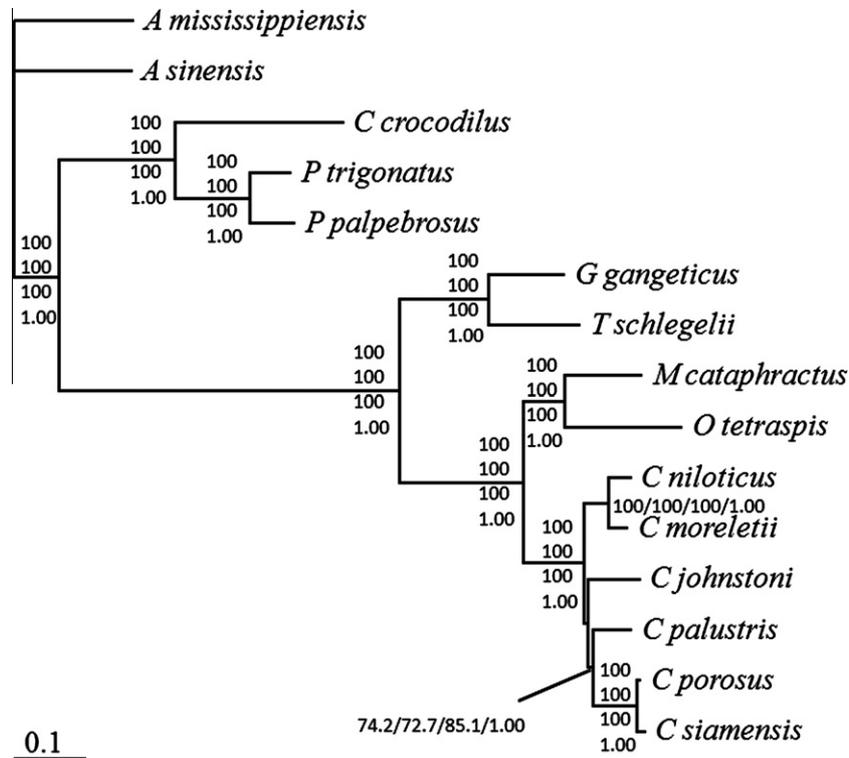


Fig. 1. Consensus Bayesian tree obtained from whole mt-genome analyses illustrating the close relationship between *C. porosus* and *C. siamensis*. The values near the nodes are bootstrap supports based on NJ, MP, ML analyses followed by Bayesian posterior probabilities. Support values below 50% are not shown.

agree to the presence of an Indopacific lineage. The position of *M. cataphractus* is noteworthy and indicates its closer relationship to *Osteolaemus* than to *Crocodylus*. In all analyses three relationships were concurrent within *Crocodylus*: (1) the sister group relationship between *C. porosus* and *C. siamensis* with high support (bootstrap: >97.8; p : 1.00), (2) the statistically well supported association between *C. novaeguineae* and *C. mindorensis* (bootstrap: >87.8; p : 1.00), (3) the association of *C. acutus* with *C. intermedius* with moderate to high statistical support (Bootstrap: >62.8; p : 0.96). The NJ, MP and Bayesian analyses show the close relatedness of *C. rhombifer* to *C. moreletii* with weak statistical support.

3.3. ND4-control region data analyses

Approximately a 703 bp ND4 and 422 bp control region sequences were combined to form a 1126 bp dataset. A repetitive and heteroplasmic region exists at the 3' end of the control region (Ray and Densmore, 2003). This portion was excluded from the analyses. This dataset consists of 514 constant, 441 parsimony informative and 612 variable sites. Closest distance, 2.4% was observed for *C. porosus*–*C. siamensis* relationship whereas *C. johnstoni* shown to be distant relative to *C. moreletii* within *Crocodylus*.

This dataset consists of all the six Indopacific crocodile species and supports the monophyly of Indopacific crocodilians. The result is presented in Fig. 3 with statistical support values. Two sister group relationships were consistently obtained herein: *C. porosus*–*C. siamensis* with strong branch support (bootstrap = 100; posterior probability = 1.00) and *C. mindorensis*–*C. novaeguineae* (bootstrap > 91.3; Bayesian probability = 1.00). All the analyses except Bayesian placed *C. johnstoni* at the basal position to the clade consist of *C. mindorensis*–*C. novaeguineae* and *C. palustris* was found to be the basal group to *C. porosus*–*C. siamensis*.

3.4. ND6-tRNA^{glu}-cyt b-ND4-control region data analysis

To corroborate the results obtained from above two datasets, these sequences were concatenated to form a separate dataset. The result of this 1426 bp analyses also agrees to the monophyly of Indopacific crocodilians and the result is shown in Fig. 4. The concordance was obtained in the sister relationship between *C. porosus* and *C. siamensis* and the close relationship of *C. novaeguineae* to *C. mindorensis* with high statistical support. This dataset also clearly resolved the position of *Mecistops* as a distant relative to *Crocodylus*.

3.5. 12S rRNA-cyt b data analyses

A 267 bp 12S rRNA and 843 bp *cyt b* gene sequences was concatenated and included in the analyses. After removing ambiguous positions, this dataset reveals 567 constant, 433 parsimony informative and 536 variable sites. The uncorrected pairwise distances obtained between *C. acutus* and *C. intermedius* was 1.3% and seen to be lowest while the highest distance was obtained between *C. siamensis* and *C. niloticus* (10.3%).

The results provided identical topologies in all analyses (Fig. 5). The dataset recognized the monophyly of genus *Crocodylus* and illustrated the presence of New World and Indopacific crocodilian lineage within *Crocodylus*. The close proximity of *C. porosus* to *C. siamensis*, *C. acutus* to *C. intermedius* and *C. moreletii* to *C. rhombifer* were concurrent in all the analyses. Besides this, the close relationship of *C. niloticus* to the clade consists of *C. acutus* and *C. intermedius* was also noticed and the *C. rhombifer*–*C. moreletii* clade was found to be basal to this relationship. Whereas the NJ, MP and ML analyses show the close relationship of *C. palustris* to the clade consist of *C. porosus* and *C. siamensis* and *C. johnstoni* was basal to this relationship.

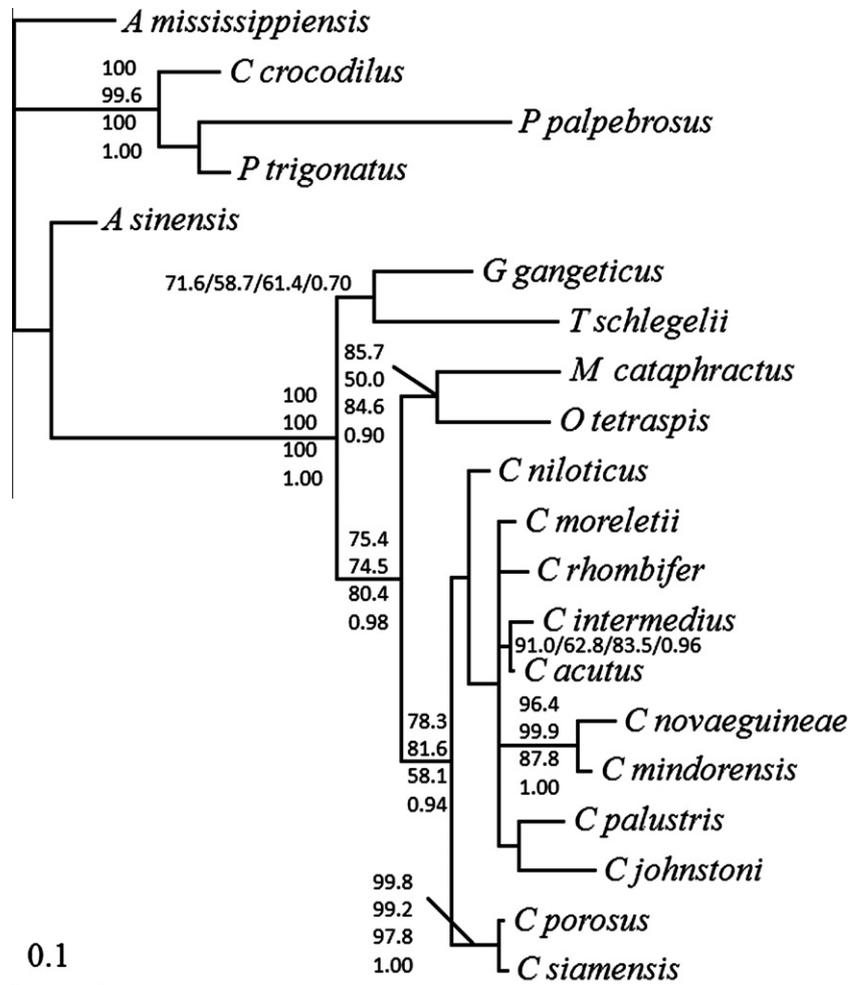


Fig. 2. Bayesian analysis tree for ND6-tRNA^{glu}-cyt *b* dataset with bootstrap values obtained from NJ, MP, ML analyses followed by posterior probabilities. Statistical support values below 50% are not shown.

3.6. Nuclear gene *C-mos* data analyses

The 368 bp sequence of this dataset consists of 338 constant, 30 variable and 19 parsimony informative characters. The partial gene sequence is highly conserved and the uncorrected pairwise distances between closely related species (*C. porosus*–*C. siamensis*; *C. acutus*–*C. intermedius*) is 0.00%. The alignment result of this data is noteworthy and shows no variation between *C. porosus* and *C. siamensis*. Whereas one base pair deletion at 23rd position and a transition at 66th position was observed in *C. palustris*. This nuclear dataset supports the established crocodylian interfamilial relationships with strong statistical support while low statistical support was obtained for closely related species. However, the results show the close relationship between *C. porosus* and *C. siamensis* and the tree is shown in Fig. 6.

Although, various data sets resulted in different levels of resolution of the phylogenetic relationships between *Crocodylus* species, a consistent placement was recovered for *C. porosus* as a sister taxon to *C. siamensis*, and a constant association of *C. acutus* with *C. intermedius* was also observed in all analyses.

4. Discussion

In recent years, the crocodylian phylogeny has been scrutinized by many authors, thus providing some resolution for the phylogenetic positions of some crocodile species, for example, *G. gangeticus*

as a close relative to *Tomistoma schlegelii* (Janke et al., 2005) and *M. cataphractus* as a distant relative to the genus *Crocodylus* (Brochu, 2000; McAliley et al., 2006). However, the relationships among the members of genus *Crocodylus* remain poorly understood and the recent studies suggest a reanalysis of crocodylian phylogeny using whole mt-genome sequences. Hence, this study was aimed to reconstruct the crocodylian phylogeny based on whole mtDNA sequences with a special reference to the phylogenetic position of saltwater crocodile, *C. porosus*. In this study the newly sequenced whole mt-genome of *C. palustris*, *C. johnstoni* and the mt-genome sequence of *C. moreletii*, used in the previous study, were analyzed along with the mt-genome sequence of other crocodile species. In order to avoid the stochastic errors arising due to single gene or a single method, different gene sequences along with different phylogenetic methods were used. These gene sequences were concatenated in the form of different datasets, as the concatenation of gene sequences is known to result in improved accuracy of the phylogenetic tree (Gadagkar et al., 2005).

4.1. Intrageneric relationships within *Crocodylus*

The whole mt-genomic analyses along with the partial mtDNA and a nuclear gene sequence examinations, provided better insights into the crocodylian phylogeny. This study sustains the monophyletic assemblage of *Crocodylus*, as evident from the morphological (Brochu, 2000) and molecular data studies (Poe, 1996;

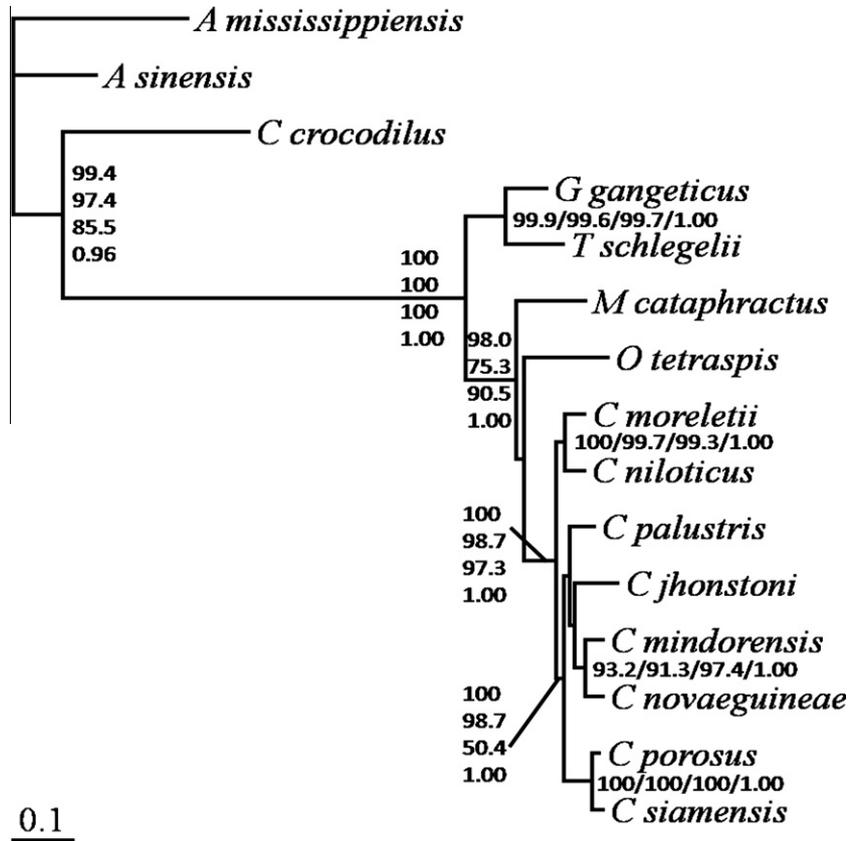


Fig. 3. Phylogenetic analyses based on ND4-control region data showing sister relationship between *C. porosus* and *C. siamensis* within Indopacific clade with strong nodal support. Only the support values above 50% obtained from NJ, MP, ML and Bayesian inferences are shown near the nodes.

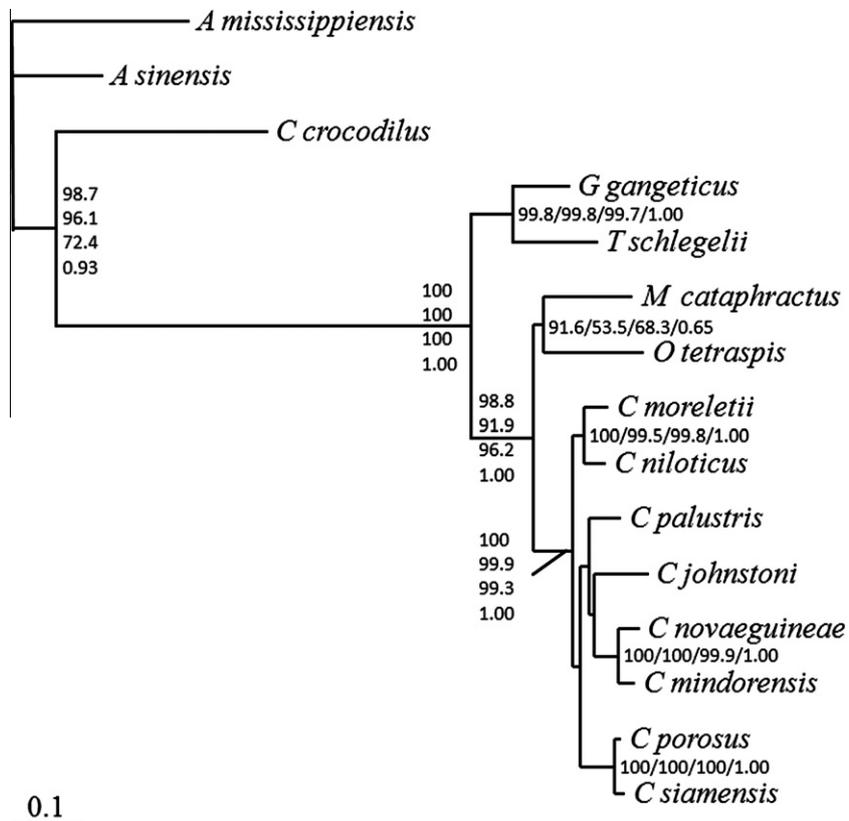


Fig. 4. Bayesian tree based on ND6-tRNA^{glu}-cyt b-ND4-control region data with bootstrap support obtained from NJ, MP, ML and Bayesian analyses. The values below 50% are not shown.

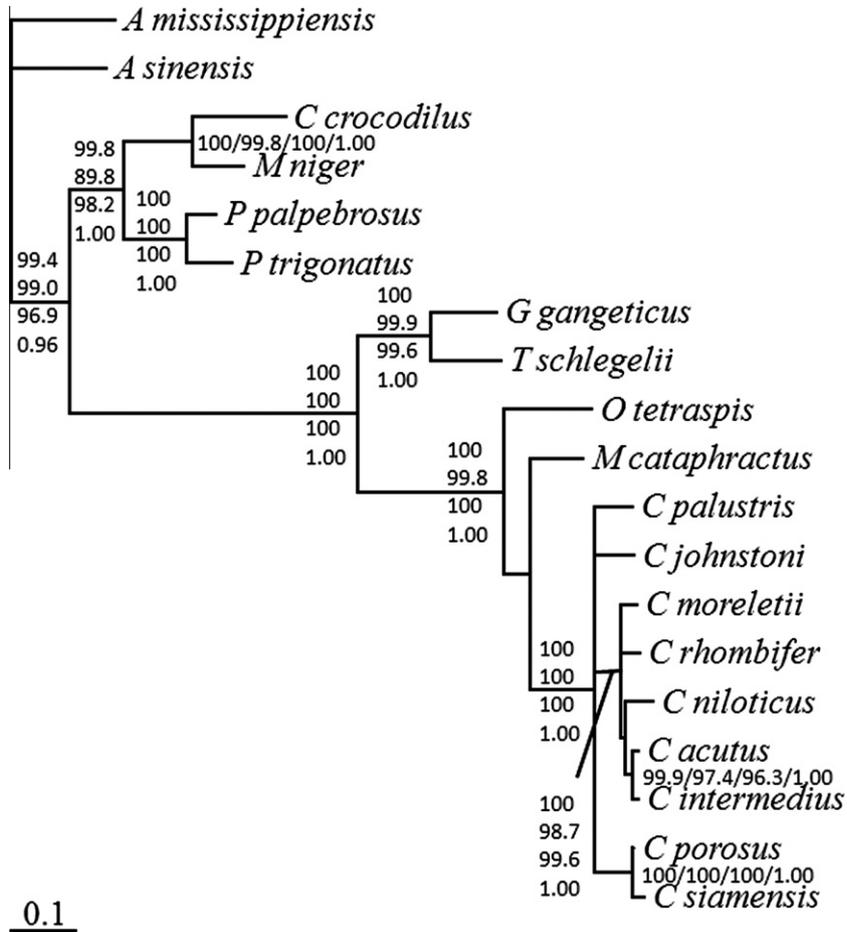


Fig. 5. Consensus Bayesian tree obtained from 12S rRNA-cyt *b* gene sequences. The NJ, MP, ML bootstrap values followed by Bayesian probabilities are shown near the nodes. Values below 50% are not given.

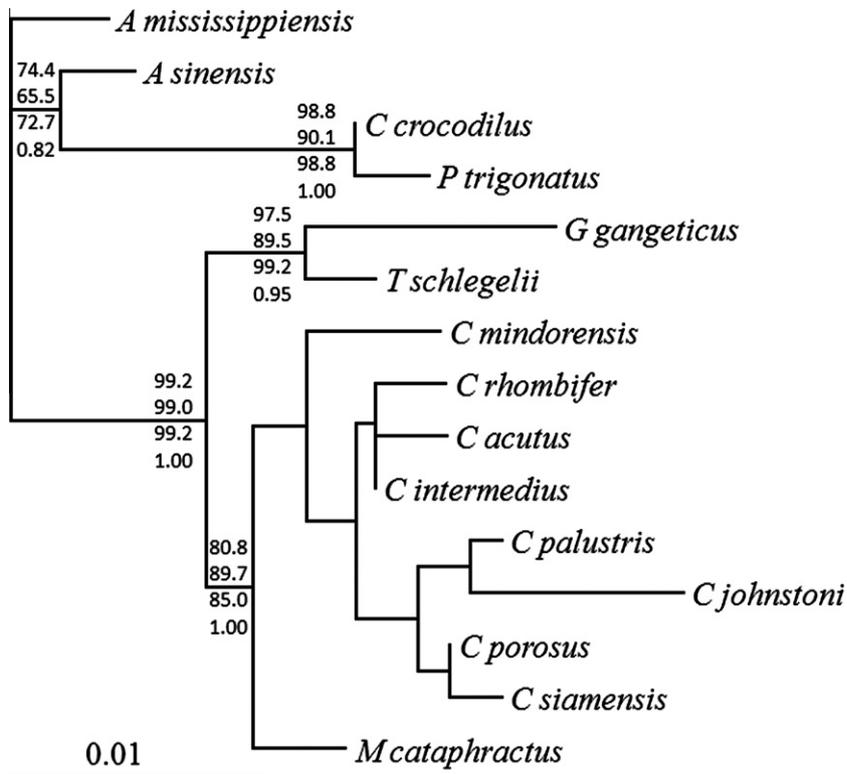


Fig. 6. Phylogenetic tree based on C-mos gene with Bootstrap values obtained from NJ, MP, ML analyses followed by posterior probabilities. Support values below 50% are not shown.

McAliley et al., 2006; Li et al., 2007; Gatesy and Amato, 2008). Many of our data sets retrieved the New World crocodylian assemblage, but the Indopacific assemblage was not consistently recovered except in analyses of 12S rRNA–cyt *b* dataset. This was also depicted in the phylogenetic study conducted by Ray (2002). The current understanding of the relationships between the members of *Crocodylus* is sparse as the divergences within *Crocodylus* are very recent (Brochu et al., 2010; Delfino and De Vos, 2010) which has probably been a problem in resolving the phylogeny within this genus. On the other hand the uncorrected pairwise genetic distances obtained herein, provided a good resolution for some relationships within genus *Crocodylus*. Earlier morphological and molecular data analyses did not provide a consistent placement for *C. porosus*, whereas our whole mt-genome analyses reveal a closer affinity of *C. porosus* to *C. siamensis* than towards any other *Crocodylus* species and this relationship was supported by all the data sets, including the best tree as evaluated by SH test for all six data sets. Although, the addition of taxa may affect tree topology (Krüger and Gargas, 2006), the absence of two species, *C. novaeguineae* and *C. mindorensis*, did not adversely affect our results as evident from the consistent placement of *C. porosus* as a sister taxon to *C. siamensis* in all the datasets analyzed, regardless of the number of taxa used in various dataset. The phylogenetic analyses by McAliley et al. (2006) including *C. novaeguineae* and *C. mindorensis* using different data sets denoted the similar relationship between *C. porosus* and *C. siamensis*, which adds further support to our findings. Moreover the *C. porosus*–*C. siamensis* relationship was also sustained in the nuclear gene analyses but with low nodal support, which is similar to the results of McAliley et al. (2006). Weak support from C-mos gene is not unexpected as the C-mos gene is highly conserved (Butorina and Colovenchuk, 2004; Godinho et al., 2006; McAliley et al., 2006). Furthermore, the recent examinations of Willis (2009) illustrate the difficulties to elucidate the relationships within *Crocodylus* using the nuclear gene, Transferrin and the reason was well explained in his study. Nevertheless, the partial C-mos gene sequences of *C. porosus* and *C. siamensis* did not show any variation in sequence alignment. However, the recent studies revealed the presence of cryptic species and cross species hybridization in crocodylians including *C. porosus* (Gratten, 2003; Ray et al., 2004; Cedeno-Vazquez et al., 2008; Rodriguez et al., 2008; Weaver et al., 2008; Eaton et al., 2009; Hekkala et al., 2009). This may elucidate erroneous phylogenetic position for a species, if it is an offspring of a hybridization event. But the current study includes the partial gene sequences of *C. porosus*, having known Indian origin and the remaining data retrieved for the analyses has been used for various crocodylian genetic studies and thus the results obtained using these sequences sound trustworthy. Hence we suggest that the saltwater crocodile, *C. porosus* is a sister taxon to Siamese crocodile, *C. siamensis*. This study also sustains the sister relationship between *C. novaeguineae* and *C. mindorensis* as reported earlier (Densmore and White, 1991; Poe, 1996; White and Densmore, 2000; Ray, 2002; Gratten, 2003; McAliley et al., 2006; Oaks, 2007; Gatesy and Amato, 2008).

Another consistent sister relationship between *C. acutus* and *C. intermedius* was also recovered within the genus *Crocodylus* in the partial mtDNA sequence analyses showing moderate to high statistical support. Similar relationship was shown in the analyses by Gatesy and Amato (2008) using 14 combined (both morphological and molecular) data sets. In addition, all tree topologies obtained from concatenated ND6–tRNA^{glu}–cyt *b* and 12S rRNA–cyt *b* data have shown the sister group relationship between *C. moreletii* and *C. rhombifer* with a low support which corroborates with the results of Gatesy and Amato (2008).

Our results indubitably show a consistent placement for *C. porosus* and *C. siamensis* as a sister group and also retrieve the sister relation of *C. acutus* with *C. intermedius*, however, the present

examinations could not conclusively place *C. palustris* and *C. johnstoni* and could not establish the nearest relative to *C. porosus* and *C. siamensis*. This could be due to the limitations of datasets with the missing taxa, *C. novaeguineae* and *C. mindorensis*. The positions of rest of the members of *Crocodylus* however, remain inconsistent and need further investigations.

4.2. Intergeneric crocodylian relationships

The reconstruction of crocodylian phylogeny including new mt-genome sequences substantiates many historically important reports. This study was concurrent with the established positions, regarding the intergeneric relationships of crocodylians. There was a clear demarcation of Crocodylia into Alligatoridae and Crocodyliidae. All the analyses including *M. cataphractus* show its distant relatedness to *Crocodylus* and placed outside the *Crocodylus* clade. Thus this study supports the results of McAliley et al. (2006) and provides clear evidence for *Mecistops* as a non-*Crocodylus* member within Crocodylia. The earlier analyses based on morphological and molecular data have shown the close relationship of *Osteolaemus* to *Crocodylus* (Steel, 1973; Salisbury and Willis, 1996; Brochu, 1997; Roos et al., 2007), which is also supported here. The analyses carried out by Janke et al. (2005) illustrate the sister relationship between *Gavialis* and *Tomistoma*, and the inclusion of new mt-genome data (in this study) did not alter this sister group relationship. In addition, the 12S rRNA–cyt *b* dataset retained the close relationship of *Caiman* and *Melanosuchus* as presented by Brochu (1997, 2003) and Harshman et al. (2003).

This is the first report on intrageneric crocodylian phylogenetics involving whole mt-genome sequences. The six data sets utilized in the present study, with extensive analysis, provide new insights into the crocodylian phylogeny. Our results gained substantial support for the intergeneric relationships as established in the previous molecular studies. This study also provides additional information for a better understanding of phylogenetic relationships between the members of genus *Crocodylus*, and strengthens the existing crocodylian phylogenetics. While many of the phylogenetic relationships within the genus *Crocodylus* are unknown, our study proposes the saltwater crocodile, *C. porosus* as a sister taxon to *C. siamensis* and also illustrates the sister group relationship between *C. acutus* and *C. intermedius* within the genus *Crocodylus*. However, these results are preliminary and further studies with extensive taxon sampling are required to confirm the proposed relationships.

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