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Near-complete phylogeny of extant Crocodylia (Reptilia) using mitogenome-based data

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Species of the order Crocodylia are mostly large, predatory and semi-aquatic reptiles. Crocodylia, the closest living relatives of birds, first appeared in the Late Cretaceous period. In the present study, the complete mitochondrial (mt) genomes of 19 Crocodylia species, including two species (*Melanosuchus niger* and *Caiman yacare*) that have not been previously sequenced for mitogenomes, were processed through Illumina sequencing to offer genetic resources and compare with the mitogenomes of Crocodylia species reported previously. In addition, a high-resolution phylogenetic tree of nearly all current recognized species of Crocodylia is constructed based on mitogenomic data. Phylogenetic analyses support monophyly of three families: Alligatoridae (four genera: *Alligator*, *Caiman*, *Melanosuchus* and *Paleosuchus*), Crocodylidae (three genera: *Crocodylus*, *Mecistops* and *Osteolaemus*) and Gavialidae (two genera: *Gavialis* and *Tomistoma*). The tree topology is generally similar to previous studies. Molecular dating suggests that the first split within Crocodylia date back to the Upper Cretaceous (approx. 86.75 Mya). The estimated time to the most recent common ancestor (TMRCA) of Alligatoridae is 53.33 Mya and that of Crocodylidae and Gavialidae is 50.13 Mya, which might be closely linked to climate changes during the Late Palaeocene and Early Eocene. Additionally, this study proves that the diversification rate within Crocodylia began to increase from the Late Eocene (about 36 Mya) and two diversification peak periods of Crocodylia (0–10 Mya and 10–20 Mya) are disclosed, which is roughly consistent with the estimated crocodylian species richness through time. Combining all these clues, we can suggest that climate fluctuation may have played a decisive role in the speciation of Crocodylia.

ADDITIONAL KEYWORDS: climate change – crocodiles – diversification – genomics – mitochondrial genome.

INTRODUCTION

Due to the high rate of molecular evolution as compared to the nuclear genome (Avise, 1989), many phylogenetic analyses have relied on the use of mitochondrial DNA (mtDNA) genes (Moritz, 1994; Freitas *et al.*, 2018; Cabrera *et al.*, 2019; Leea *et al.*, 2019). Over the last two decades, mitogenomes have been exploited for the reconstruction of higher resolution phylogenies in different taxa (Ingman *et al.*, 2000; Freitas *et al.*, 2018; Bittencourt *et al.*, 2019; Cabrera *et al.*, 2019; Leea *et al.*, 2019; Pan *et al.*, 2019). Previous mitogenomic studies have widely used conventional approaches of combining long-range PCR procedure with subsequent primer walking, which is labour-intensive and costly (Huyse *et al.*, 2007; Driscoll *et al.*, 2009). With the development of new technology, the more straightforward approach for reconstructing novel mitogenomes directly from next-generation sequencing (NGS) data, derived from total genomic DNA extracts, has become increasingly important and influential in this field (Hahn *et al.*, 2013; Han *et al.*, 2014; Mitchell *et al.*, 2014). Phylogenetic analyses

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have also been greatly advanced by the increase in genetic and genomic resources, which has prompted an increased interest in mitogenomes (Freitas *et al.*, 2018; Vanhove *et al.*, 2018; Ma *et al.*, 2019; Pons *et al.*, 2019).

Crocodylia is an order comprised of mostly large, predatory, semi-aquatic reptiles. As a surviving member of the Archosauria, they first appeared in the Late Cretaceous period and remain the closest living relatives of birds (Green *et al.*, 2014). Until recently, 23 species in eight genera were accepted in extant Crocodylia, based on classical morphological taxonomy (Oaks, 2011). However, with more fieldwork and a combination of molecular and morphological approaches, it has become clear that some of these species are better subdivided or reassigned (Brochu, 2007; Eaton *et al.*, 2009; Hekkala *et al.*, 2011; Shirley *et al.*, 2014). In the *Biology and evolution of crocodylians*, Grigg & Kirshner (2015) concluded that the order Crocodylia is comprised of 23 recognized and four proposed living species distributed among nine genera (*Alligator*, *Caiman*, *Crocodylus*, *Gavialis*, *Mecistops*, *Melanosuchus*, *Osteolaemus*, *Paleosuchus* and *Tomistoma*) placed in three families (Alligatoridae, Crocodylidae and Gavialidae). These four proposed species included one Nile crocodile (*Cr. suchus* Geoffroy Saint-Hilaire, 1807) (Hekkala *et al.*, 2011), two dwarf crocodiles (*O. osborni* Cope, 1861 and a probable third species) (Brochu, 2007; Eaton *et al.*, 2009; Shirley *et al.*, 2014) and one cryptic species in *Mec. cataphractus* Cuvier, 1825 (Shirley *et al.*, 2014). Since 2015, several other studies on species delimitation or population genetics in the order Crocodylia were carried out (e.g. Bloor *et al.*, 2015; Muniz *et al.*, 2018; Shirley *et al.*, 2018; Bittencourt *et al.*, 2019; Murray *et al.*, 2019). Among these studies, the cryptic species in *Mec. cataphractus* showed the revalidation of *Mec. leptorhynchus* (Bennett, 1835), based on the morphological divergence and phylogenetic placement (Shirley *et al.*, 2014, 2018). Cuvier's dwarf caiman *P. palpebrosus* (Cuvier, 1807) comprises at least three lineages, suggesting that *P. palpebrosus* is also a species complex (Muniz *et al.*, 2018). Schneider's dwarf caiman *P. trigonatus* (Schneider, 1801) has two well-supported lineages, which also indicates potential branch diversity (Bittencourt *et al.*, 2019). In Papua New Guinea, the freshwater crocodile *Cr. novaeguineae* Schmidt, 1928 includes two divergent northern and southern lineages on either side of the Central Highlands due to morphological, molecular and ecological divergence, with the southern lineages now formally described as a new species, Hall's New Guinea crocodile, *Cr. halli* Murray *et al.*, 2019 (Murray *et al.*, 2019). These Crocodylia lineages are independent of being identified as different species or having been formally described, but are a direct outcome of evolution. Therefore, further research is necessary to disclose the evolutionary history within the order Crocodylia. Recent studies using whole mitogenomes within Crocodylia have shown that mitogenomes may be more effective in defining the relationships among some species as compared to phylogenetic analyses based on shorter mitochondrial fragments (Roos *et al.*, 2007; Feng *et al.*, 2010; Meganathan *et al.*, 2010; Meredith *et al.*, 2011; Oaks, 2011; Zhang *et al.*, 2011; Milián-García *et al.*, 2018). To date, the mitochondrial genome data for nearly all species of Crocodylia, except for two species [*Mel. niger* (Spix, 1825) and *Ca. yacare* (Daudin, 1802)], has been available in the NCBI database. Therefore, the mitochondrial genome of these two species still needs to be sequenced and analysed. Additionally, a systematic study based on all existing mitogenomic data in Crocodylia is still needed to construct a phylogeny that will provide a robust explanation for the phylogenetic relationships within classical Crocodylia taxonomy.

In this study, we characterize a high-quality, near-complete, mitochondrial genome of *Mel. niger* and *Ca. yacare*, respectively, using Illumina short-read sequencing, and perform phylogenomic analyses of Crocodylia order using 58 complete or near-complete mitogenomes in an effort to construct a more robust backbone phylogeny and to evaluate relationships within and among each major clade. Through mitogenomic investigation and phylogenomic analysis of the order Crocodylia, we address the following objectives: (1) to characterize the newly sequenced mitochondrial genome, (2) to determine the phylogenetic position of the two newly sequenced species (*Mel. niger* and *Ca. yacare*) and (3) to explore implications for phylogenetic relationships and speciation processes in Crocodylia.

MATERIALS AND METHODS

Sample preparation

The blood samples of 19 individuals belong to 19 species (see Table 1) of Crocodylia were collected from the laboratory of Anhui Normal University (presented by Dr George Amato from AMNH). These samples were stored at –80 °C. We extracted the total genomic DNA by the DNeasy Blood and Tissue Kit (Qiagen, USA). For detailed library building (read length of each pair-end library, 150 bp) and sequencing information, see the Supporting Information (Figs S1–S4) and methods. The basic statistics of sequencing data is summarized in (Table 2). The raw Illumina data were filtered and trimmed using the script IlluQC.pl within NGS_QC_Toolkit v.2.3.3 (Concolino *et al.*,

Table 1. The sources of mitogenomes in Crocodylia and outgroups utilized in this study. GeneBank accession numbers and genome length are included. The sample numbers for the new individuals (e.g, L062, L015, etc.) are listed behind the Latin name. The genome length of some species does not include the control region

Order	Family	Genus	Latin Name	Accession Number	Length (bp)	Source
Crocodylia	Alligatoridae	Alligator	*1.Alligator sinensis*	AF511507	16 746	Unpublished
			2.Alligator mississippiensis	Y13113	16 646	(Janke & Arnason, 1997)
			3.Alligator mississippiensis	AF069428	15 898	Unpublished
		Melanosuchus	*4.Melanosuchus niger* L062	MT554039	15 918	This study
			5.Caiman latirostris L015	MT554051	16 558	This study
		Caiman	*6.Caiman crocodilus*	AJ404872	17 900	(Janke *et al.*, 2001)
			7.Caiman crocodilus L024	MT554050	16 558	This study
			8.Caiman yacare L070	MT554042	16 553	This study
		Paleosuchus	*9.Paleosuchus palpebrosus*	AM493870	14 935	(Roos *et al.*, 2007)
			10.Paleosuchus palpebrosus	KJ920382	16 603	Unpublished
			11.Paleosuchus palpebrosus	NC009729	14 935	(Roos *et al.*, 2007)
			12.Paleosuchus palpebrosus L101	MT554041	16 976	This study
			13.Paleosuchus trigonatus	AM493869	15 948	(Roos *et al.*, 2007)
			14.Paleosuchus trigonatus L028	MT554034	17 708	This study
	Crocodylidae	Crocodylus	*15.Crocodylus intermedius*	JF502242	16 169	(Meredith *et al.*, 2011)
			16.Crocodylus intermedius	HM636895	16 828	(Zhang *et al.*, 2011)
			17.Crocodylus intermedius L048	MT554044	17 094	This study
			18.Crocodylus acutus	JF502241	16 168	(Meredith *et al.*, 2011)
			19.Crocodylus acutus	NC015647	16 883	(Zhang *et al.*, 2011)
			20.Crocodylus acutus L014	MT554043	17 438	This study
			21.Crocodylus rhombifer	JF502247	16 167	(Meredith *et al.*, 2011)
			22.Crocodylus rhombifer	JX292787	16 795	Unpublished
			23.Crocodylus rhombifer L032	MT554036	16 640	This study
			24.Crocodylus moreletii	HQ585889	16 827	(Meganathan *et al.*, 2011)
			25.Crocodylus moreletii L086	MT554046	15 729	This study
			26.Crocodylus niloticus 'eastern'	JF502246	16 168	(Meredith *et al.*, 2011)
			27.Crocodylus niloticus	DQ273697	16 776	(Ji *et al.*, 2006)
			28.Crocodylus niloticus 'eastern'	JF502245	16 168	(Meredith *et al.*, 2011)
			29.Crocodylus niloticus	NC008142	16 830	(Janke *et al.*, 2005)
			30.Crocodylus niloticus	AJ810452	16 830	(Janke *et al.*, 2005)
			31.Crocodylus niloticus "western"	JF502243	16 172	(Meredith *et al.*, 2011)
			32.Crocodylus niloticus 'western'	JF502244	16 172	(Meredith *et al.*, 2011)
			33.Crocodylus niloticus L050	MT554047	16 760	This study
			34.Crocodylus palustris	GU144286	17 052	(Feng *et al.*, 2010)
			35.Crocodylus palustris	HM488007	16 852	(Meganathan *et al.*, 2011)
			36.Crocodylus palustris	NC014706	17 052	(Feng *et al.*, 2010)
			37.Crocodylus palustris L089	MT554049	17 464	This study

Table 1. Continued

Order	Family	Genus	Latin Name	Accession Number	Length (bp)	Source
			38.Crocodylus porosus	NC008143	16 916	(Janke *et al.*, 2005)
			39.Crocodylus porosus	AJ810453	16 916	(Janke *et al.*, 2005)
			40.Crocodylus porosus	DQ273698	16 917	(Li *et al.*, 2007)
			41.Crocodylus siamensis	EF581859	16 814	Unpublished
			42.Crocodylus siamensis L019	MT554035	16 933	This study
			43.Crocodylus novaeguineae	JF502240	16 152	(Meredith *et al.*, 2011)
			44.Crocodylus novaeguineae	HM636896	16 894	(Zhang *et al.*, 2011)
			45.Crocodylus novaeguineae L088	MT554048	17 763	This study
			46.Crocodylus mindorensis	GU144287	16 939	(Feng *et al.*, 2010)
			47.Crocodylus mindorensis L080	MT554045	17 865	This study
			48.Crocodylus johnstoni	NC015238	16 851	(Meganathan *et al.*, 2011)
			49.Crocodylus johnstoni L035	MT554037	17 316	This study
			50.Mecistops cataphractus	NC010639	16 847	Unpublished
			51.Mecistops cataphractus L013	MT554033	17 316	This study
		Osteolaemus	*52.Osteolaemus tetraspis*	NC009728	16 814	(Roos *et al.*, 2007)
			53.Osteolaemus tetraspis	AM493868	16 814	(Roos *et al.*, 2007)
			54.Osteolaemus tetraspis	EF551001	16 873	Unpublished
			55.Osteolaemus tetraspis L045	MT554038	17 795	This study
	Gavialidae	Tomistoma	*56.Tomistoma schlegelii*	AJ810455	16 345	(Janke *et al.*, 2005)
			57.Tomistoma schlegelii L065	MT554040	16 872	This study
		Gavialis	*58.Gavialis gangeticus*	AJ810454	17 023	(Janke *et al.*, 2005)
Outgroups						
Carinatae	Phasianidae	Gallus	*59.Gallus gallus*	AP003580	16 788	(Nishibori *et al.*, 2003)
			60.Gallus gallus	AP003317	16 788	(Nishibori *et al.*, 2003)
			61.Gallus gallus	AP003318	16 785	(Nishibori *et al.*, 2003)
			62.Gallus gallus	AB086102	16 784	(Wada *et al.*, 2004)
Struthionformes	Struthionidae	Struthio	*63.Struthio camelus*	AF338715	16 595	(Haddrath & Baker, 2001)
			64.Struthio camelus	NC002785	16 595	(Haddrath & Baker, 2001)
			65.Struthio camelus	Y12025	16 591	(Härlid *et al.*, 1997)
Columbiformes	Columbidae	Columba	*66.Columba livia*	KP258178	17 221	(Zhang *et al.*, 2015a)
			67.Columba livia	GU908131	17 229	(Kan *et al.*, 2010)
			68.Columba livia	KP168712	17 233	(Zhang *et al.*, 2015b)
Sphenisciformes	Sphenisciformes	Aptenodytes	*69.Aptenodytes forsteri*	NC027938	17 301	Unpublished
Passeriformes	Estrildidae	Taeniopygia	*70.Taeniopygia guttata* haplotype D	DQ453513	16 854	(Mossman *et al.*, 2006)
			71.Taeniopygia guttata haplotype E	DQ453514	16 854	(Mossman *et al.*, 2006)
			72.Taeniopygia guttata	DQ422742	16 853	(Mossman *et al.*, 2006)
			73.Taeniopygia guttata	NC007897	16 853	(Mossman *et al.*, 2006)

Table 1. Continued

Order	Family	Genus	Latin Name	Accession Number	Length (bp)	Source
			74.Taeniopygia guttata haplotype B	DQ453515	16 853	(Mossman *et al.*, 2006)
			75.Taeniopygia guttata haplotype C	DQ453512	16 853	(Mossman *et al.*, 2006)
Tesudines	Cheloniidae	Chelonia	*76.Chelonia mydas*	AB012104	16 497	(Kumazawa & Nishida, 1995)
			77.Chelonia mydas	JX454990	16 495	(Duchene *et al.*, 2012)
			78.Chelonia mydas	JX454972	16 440	(Duchene *et al.*, 2012)
			79.Chelonia mydas	JX454971	16 435	(Duchene *et al.*, 2012)
			80.Chelonia mydas	JX454974	16 435	(Duchene *et al.*, 2012)
			81.Chelonia mydas	JX454976	16 435	(Duchene *et al.*, 2012)
	Trionychidae	Pelodiscus	*82.Pelodiscus sinensis*	AY687385	17 364	Unpublished
			83.Pelodiscus sinensis	AY962573	17 042	(Jung *et al.*, 2006)
Squamata	Boidae	Python	*84.Python molurus molurus*	HM581978	17 258	Unpublished
			85.Python bivittatus	NC021479	17 617	Unpublished
	Agamidae	Anolis	*86.Anolis carolinensis*	NC010972	17 223	Unknown

2014). FastUniq software (Xu *et al.*, 2012) was used to remove the duplicated reads.

MITOGENOME ASSEMBLY AND CHARACTERIZATION

To obtain the mitochondrial genome sequence of the best quality for each sample, both reference dependent and reference independent mitogenome assembly strategies were taken. Here, we employed the software MITObim v.1.9.1 (Hahn *et al.*, 2013) and SOAPdenovo v.2 (Luo *et al.*, 2012) to do the mitogenome assembly based on the Illumina data. For detailed mitogenome assembly information and evaluation of assembly quality, see the the Supporting Information (Figs S1–S4) and methods. The candidate mitochondrial genome sequence in our *de novo* assemblies will be identified by blast search with criteria that the similarity value should be over 90% and the length of subject contig/scaffold should not be less than 85% of the query sequence (Camacho *et al.*, 2008). The mitogenomes with higher continuity and higher similarity with corresponding previously released sequences was kept to represent the mitogenomes for this sample.

Sequences were aligned using Clustal X v.2.0 (Larkin *et al.*, 2007). The known complete mtDNA sequences of *Ca. crocodilus* (Linnaeus, 1758) (AJ404872) were used to identify protein-coding genes, and the 22 tRNA genes were identified by tRNA Scan-SE v.1.21 (http://lowelab.ucsc.edu/tRNAscan-SE 1.2.1). All assembled and annotated mitogenomes were submitted to GenBank (MT554033–MT554051; Table 1).

MITOCHONDRIAL PHYLOGENY

In this study, we used 86 mitogenomes to reconstruct the phylogenetic tree between taxa without partitions using Bayesian inference (BI) and maximum likelihood (ML) (see Table 1), including 19 newly generated mitogenomes of Crocodylia, 39 mitogenomes of 21 Crocodylia species publicly available in NCBI and, additionally, 28 mitogenomes of ten other species (two pythons, one lizard, five birds and two turtles; Green *et al.*, 2014). All alignment-ambiguous regions and control regions were removed to avoid erroneous phylogenetic hypotheses, and alignment gaps were analysed as missing data. The best-fit DNA sequence evolution model of our dataset was estimated with jModeltest v.2 using the Bayesian information criterion (BIC) to choose the most suitable model (Darriba *et al.*, 2012). The Bayesian phylogenetic tree was inferred using MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001) and the best-fit model identified with jModeltest. Two independent runs of the Markov chain Monte Carlo (MCMC) algorithm were performed to assess convergence of posterior probability distributions. The run parameters used were set to 1 × 10^7 iterations of the MCMC algorithm sampled every 1000 iterations and the first 10% of the iterations were discarded as burn-in. An average standard deviation of split frequencies of 0.01 was used to check model stability. RaxML v.8 (Stamatakis, 2014) was used to perform ML analyses with a general time-reversible model of nucleotide substitution under the Gamma model of rate heterogeneity (i.e. GTRCAT), with 1000 bootstrap iterations to determine internal branch support of the best-scoring tree.

DIVERGENCE-TIME ANALYSES

To estimate divergence times between different clades of Crocodylia, we used BEAST v.1.8.0 (Drummond *et al.*, 2012) to calculate an ultrametric tree. All the calibration points (a, 260–289 Mya; b, 77.8–83.6 Mya; Fig. 2) were adopted from earlier studies (Lyson *et al.*, 2010; Dos *et al.*, 2012; Jones *et al.*, 2013; Green *et al.*, 2014). In order to ensure the stability of the tree topology, we set the crocodile and bird species as monophyletic, following the study of Green *et al.* (2014). For this analysis we used a relaxed uncorrelated log normal model of lineage variation, a Yule Process prior for the branching rates and a GTR+G model of sequence evolution (best-selected model). Four replicates of the analysis were run for 1 × 10^7 generations with parameter and tree sampling every 1000 generations, discarding the first 25% of BEAST's MCMC iterations as burn-in. Convergence between runs was monitored using Tracer v.1.7 (Rambaut *et al.*, 2018) and ESS values indicative of adequate sampling (> 200). The phylogenetic tree was generated and visualized with TreeAnnotator v.1.8.0 (Drummond et al., 2012) and FigTree v.1.4.3 (Rambaut, 2016), respectively. For visualizing diversification rate changes, we plotted the number of newly appearing species against the fixed time intervals of 10 Myr (Venditti *et al.*, 2010).

RESULTS

GENERAL FEATURES OF CROCODYLIA MITOGENOMES

High-throughput, whole-genome sequencing (WGS) projects have been widely used to provide short reads originated from not only the nucleus of both animals and plants, but also from others organelles such as mitochondria. Due to the small size of the Crocodylia mitochondrial genome (~20 kb), and abundant copies of the mitochondria segment, the sequencing depth of mitochondrial genome is often much deeper than that of nuclear DNA. These advantages contribute to making WGS data a valuable resource for mitogenome assembly. In this study, we compared both reference-dependent and reference-independent mitogenome assembly methods and the better assembly was selected based on the assembly continuity index (N50: the minimum contig/scaffold length needed to cover 50% of the assembly) and total length. Nineteen complete or near-complete mitogenomes were assembled for species of Crocodylia, especially for two species that had their genomes sequenced for the first time (*Mel. niger*, with a length of 15 922 bp; *Ca. yacare*, 15 911 bp) (Fig. 1). The annotated sequences are deposited in NCBI GenBank (Table 1). The ribosomal operons of *Mel. niger* (6799 bp) and *Ca. yacare* (7005 bp) are deposited as additional molecular vouchers for these species. The order of protein-coding genes and tRNA genes for these two species are identical, except the control region (lack of adequate data to obtain the complete sequence). To validate the quality of our mitochondrial assembly, and to detect the large genome variation, we also aligned our new assembled genome sequence to previously released mitogenomes of the same or closely related species. Most of the comparison shows great similarity represented by a single red line from the bottom left to the top right in the dot plots (Supporting Information, Fig. S1). We found that there was good mitogenome synteny among different species in *Caiman* genera, such as *Ca. latirostris* (Daudin, 1802) (L015) and *Ca. crocodilus* (GenBank: AJ404872.2), *Ca. yacare* (L070) and *Ca. crocodilus* (GenBank: AJ404872.2) (Supporting Information, Figs S2, S3). We also identified some insertions in our assembly compared to previously released sequences from other research, especially for the control region, such as *Cr. novaeguineae* (L088 and GenBank: NC015651.1). It is worth noting that some mitogenome assembled in this study is identical to the previously released mitochondrial genome sequence, including *Cr. novaeguineae* (L088 and GenBank: JF502240), *Cr. intermedius* (Graves, 1918) (L048 and GenBank: JF502242) and *Cr. rhombifer* (Cuvier, 1807) (L032 and GenBank: JF502247). Additionally, we also found that some public complete mitochondrial genome sequences were identical to each other, including *Cr. porosus* Schneider, 1801 (AJ810453 and NC008143), *Cr. niloticus* Laurenti, 1768 (AJ810452 and NC008142) and *O. tetraspis* Cope, 1861 (AM493868 and NC009728), indirectly validating our strategies of assembly in this study.

PHYLOGENETIC RELATIONSHIPS

Phylogenetic relationships among Crocodylia are inferred for a concatenated dataset (17 893 bp) of the mitochondrial genome without control regions using ML and BI methods. Tree topology results from both ML and BI analyses are identical in that the Crocodylia is subdivided into three clades (A, Crocodylidae; B, Gavialidae; C, Alligatoridae) with high support values (Fig. 1). Phylogenetic analyses support the monophyly

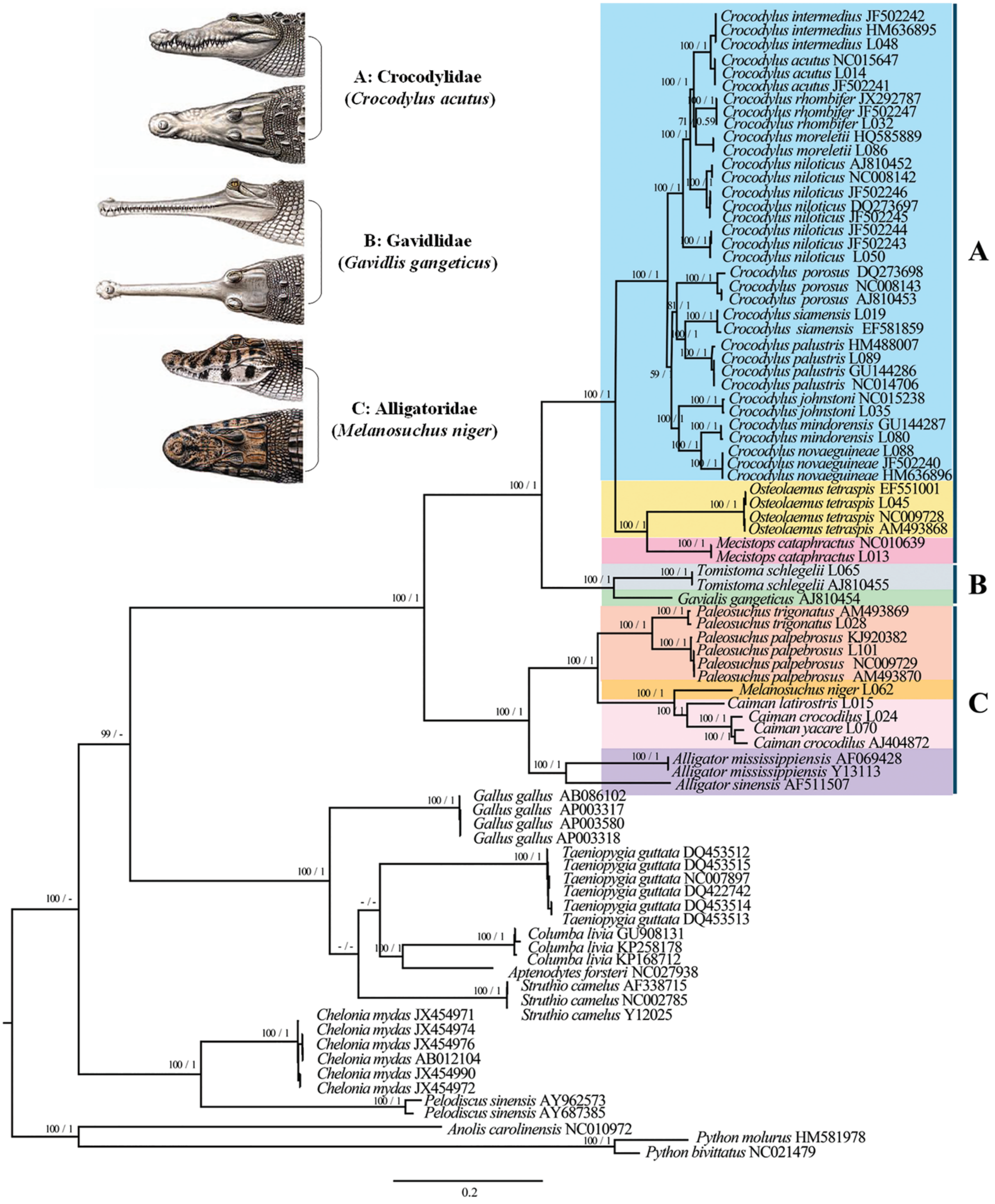

Figure 1. Maximum likelihood phylogenetic tree based on the resulting mitogenomes except control region for all known Crocodylia species. The mitogenomes employed have been identified by their accession number to the GenBank database. Ten species (two pythons, one lizard, five birds, two turtles) were used as outgroups to root the tree. The numbers on the nodes indicate ML bootstrap support (shown as a percentage) and Bayesian posterior probabilities. These letters (A, B and C) indicate the families in Crocodylia (Crocodylidae, Gavialidae and Alligatoridae). The head shapes and dentition in 'typical' examples of the three families of living Crocodylia (dorsal and side views) (Illustrations DSK, courtesy Weldon Owen Publishing)(Grigg & Kirshner, 2015).

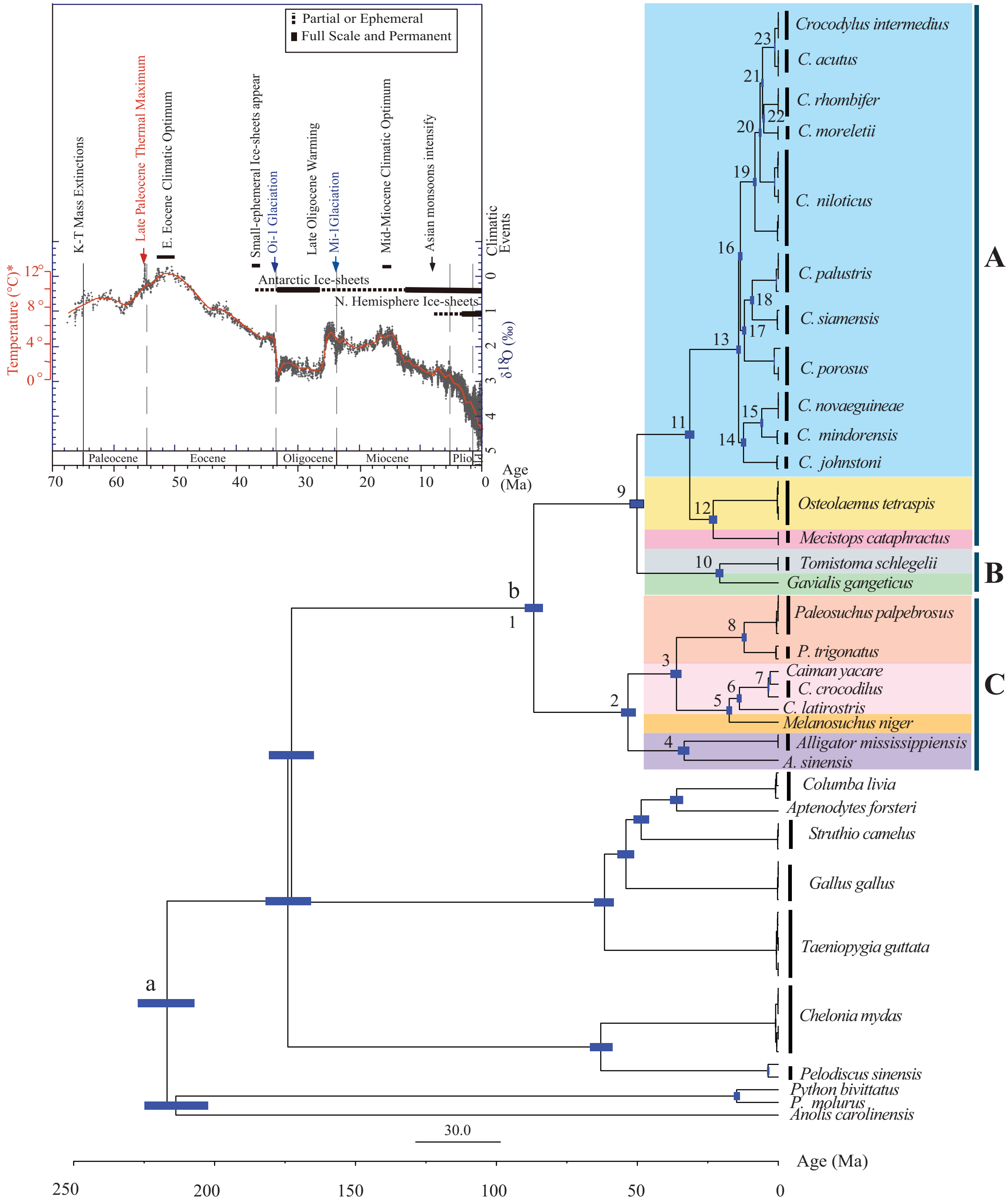

Figure 2. Time-calibrated phylogeny of the Crocodylia inferred from the complete mitogenomes. These letters (a and b) indicate the calibration points. The blue lines on nodes correspond to the 95% highest posterior density of the age of the node. The bottom axis is in millions of years (Myr). The climatic sequence of events including a global average δ^{18}O curve (right-hand axis) derived from benthic foraminifera which mirrors the major global temperature trends from the Palaeocene to the Pleistocene (Zachos *et al.*, 2001, 2008). The establishment of ice sheets in the Northern Hemisphere is indicated by grey to black bars on top. Some key tectonic are listed (Zachos *et al.*, 2001). These letters (A, B and C) indicate the families in Crocodylia (Crocodylidae, Gavialidae and Alligatoridae). The numbers on the nodes within Crocodylia indicate node numbers, corresponding to Table 3.

of the three Crocodylia families and the nine genera: Alligatoridae (*Alligator*, *Caiman*, *Melanosuchus* and *Paleosuchus*), Crocodylidae (*Crocodylus*, *Mecistops* and *Osteolaemus*) and Gavialidae (*Gavialis* and *Tomistoma*).

MOLECULAR DATING AND LINEAGE THROUGH TIME

A high ESS (effective sample size) value (> 200) is identified for all parameters in the BEAST analysis to estimate the divergence time of Crocodylia species (Fig. 2). Dating analyses suggests that the most recent common ancestor (TMRCA) of Crocodylia dates back to 86.8 Mya [mean value; 95% of the highest posterior density (HPD), 83.6–90.02 Mya] (Table 3; Fig. 2). The TMRCA of Alligatoridae was estimated at 53.39 Mya (95% HPD, 50.77–56.02 Mya). The split between Crocodylidae and Gavialidae is timed at 50.13 Mya (95% HPD, 47.50–52.56 Mya), which is roughly consistent with the previous study Oaks (2011). The visualization of lineage diversification of Crocodylia shows that, since 36.18 Mya (the TMRCA of *Caiman*, *Melanosuchus* and *Paleosuchus*), the diversification rate within this order began to increase during the Late Eocene (Fig. 3). The cumulative curve of species-birth per time interval shows that the diversification of Crocodylia fluctuated through time, especially between 0–10 Mya and 10–20 Mya (Supporting Information, Fig. S4).

DISCUSSION

PHYLOGENETIC RELATIONSHIPS WITHIN CROCODYLIA

Crocodylia appears in the biosphere as a top predator in the food chain, playing an important

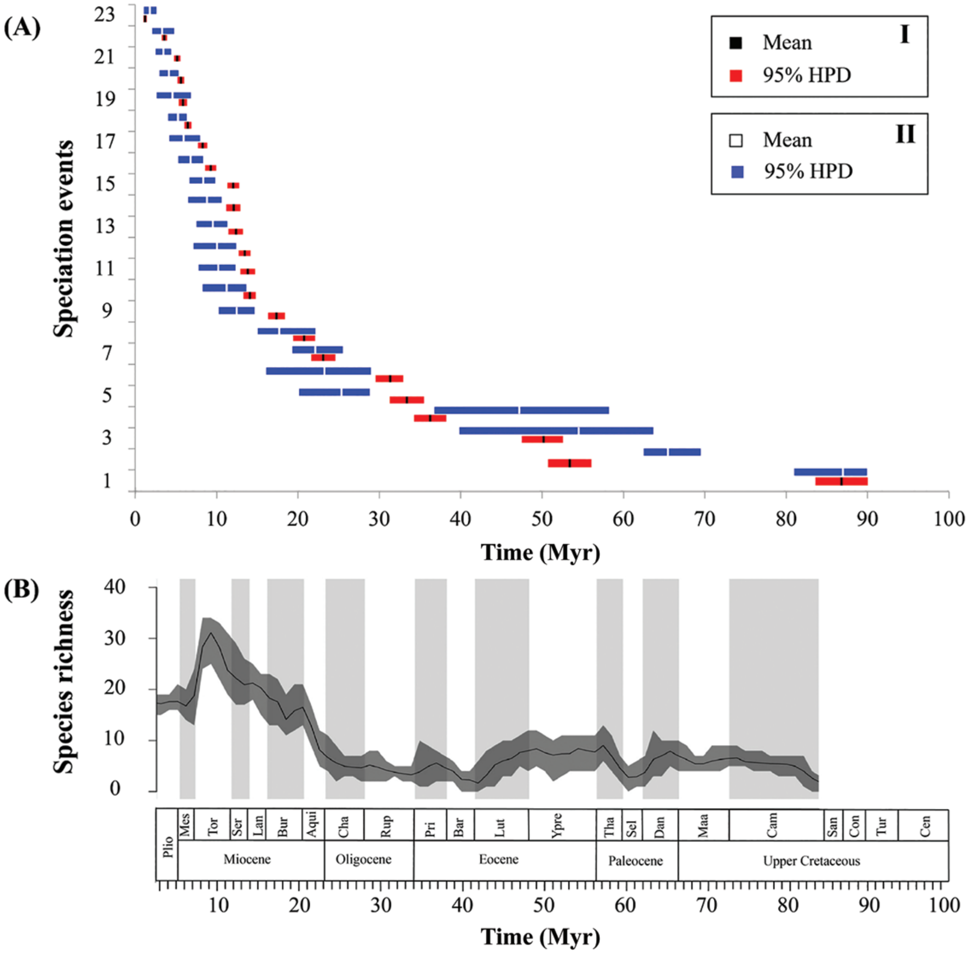

Figure 3. Visualization of lineage diversification of Crocodylia (A, B). A, credibility intervals for the age of the most recent common ancestor (TMRCA) of different lineages in the Crocodylia. I indicates the results collected from this study; II indicates the results collected from a previous study (Oaks, 2011). B, estimated crocodylian species richness through time (in Myr) (Solórzano *et al.*, 2019).

Table 2. Basic statistics for the Illumina data in the present study

Family	Species name	Total number of reads	Percentage of HQ reads	Total number of HQ filtered reads	Percentage of HQ filtered reads
Alligatoridae	1.*Melanosuchus niger* L062	242 855 387	69.92%	169 800 113	69.92%
	2.*Caiman latirostris* L015	235 597 454	82.94%	195 393 658	82.94%
	3.*Caiman crocodilus* L024	284 026 531	78.57%	223 167 902	78.57%
	4.*Caiman yacare* L070	299 730 428	76.31%	228 725 112	76.31%
	5.*Paleosuchus palpebrosus* L101	264 511 896	76.65%	202 744 280	76.65%
	6.*Paleosuchus trigonatus* L028	306 431 465	68.97%	211 354 979	68.97%
Crocodylidae	7.*Crocodylus intermedius* L048	240 909 027	83.98%	202 325 855	83.98%
	8.*Crocodylus acutus* L014	249 045 809	78.58%	195 703 045	78.58%
	9.*Crocodylus rhombifer* L032	262 260 950	83.10%	217 930 246	83.10%
	10.*Crocodylus moreletii* L086	280 339 019	68.62%	192 376 661	68.62%
	11.*Crocodylus niloticus* L050	337 207 775	82.25%	277 351 508	82.25%
	12.*Crocodylus palustris* L089	317 383 902	68.64%	217 849 013	68.64%
	13.*Crocodylus siamensis* L019	286 915 581	81.84%	234 823 316	81.84%
	14.*Crocodylus novaeguineae* L088	326 832 795	73.87%	241 414 115	73.86%
	15.*Crocodylus mindorensis* L080	292 183 230	78.96%	230 709 887	78.96%
	16.*Crocodylus johnstoni* L035	288 471 362	81.25%	234 386 237	81.25%
	17.*Mecistops cataphractus* L013	258 833 730	81.40%	210 695 089	81.40%
	18.*Osteolaemus tetraspis* L045	245 622 438	75.30%	184 956 099	75.30%
Gavialidae	19.*Tomistoma schlegelii* L065	302 765 485	80.14%	242 626 089	80.14%

role in the stability of the ecosystem. Due to the ancient and special morphology of this order, it has always attracted the attention of researchers in various fields of study. Some of these studies tended to focus on the evolutionary history within the order Crocodylia, with phylogenetic studies occupying a relatively large proportion (Brochu, 2007; Eaton *et al.*, 2009; Hekkala *et al.*, 2011; Shirley *et al.*, 2014, 2018; Bloor *et al.*, 2015; Muniz *et al.*, 2018; Bittencourt *et al.*, 2019; Murray *et al.*, 2019). Classical taxonomy of Crocodylia accept 23 extant species in this order (Oaks, 2011), although some of these species have recently been revised or still need revision (Brochu, 2007; Eaton *et al.*, 2009; Hekkala *et al.*, 2011; Shirley *et al.*, 2014, 2018; Grigg & Kirshner, 2015; Muniz *et al.*, 2018; Bittencourt *et al.*, 2019). The phylogenetic tree presented in this study supports the monophyly of the families and genera of the traditional Crocodylia families, agreeing with many recent studies (Oaks, 2011; Shirley *et al.*, 2014; Grigg & Kirshner, 2015; Nicolaï & Matzke, 2019). However, *Cr. niloticus* was not found to be monophyletic, suggesting the possible presence of a cryptic species: *Cr. suchus*. This cryptic species was first described based on similar mummified crocodiles (Geoffroy Saint-Hilaire, 1807), and was followed by application of this name for the western and central lineage of *Cr. niloticus* due to the divergent genetic difference and fixed discrete and non-overlapping continuous character variation (Hekkala *et al.*, 2011; Cunningham *et al.*, 2016). Combining these clues, we similarly suggest that basal lineage of the *Cr. niloticus* clade is most likely to be treated as an independent species *Cr. suchus*.

Melanosuchus and *Caiman* are grouped as sister-taxa. Within *Caiman*, *Ca. yacare* forms a distinct cluster with *Ca. crocodilus*, which is largely consistent with the study by Oaks (2011), with the difference that the relationship between *Ca. yacare* and *Ca. crocodilus* has not been well supported in our research. *Caiman crocodilus* forms a cluster with *Ca. yacare*, resulting in the paraphyly of *Ca. crocodilus* (Figs 1, 2). Considering our limited available number of samples (only two *Ca. crocodilus* and one *Ca. yacare*), it may be speculated that an earlier sample (AJ404872) (Janke *et al.*, 2001), was not recognized as *Ca. yacare*, or that there are additional cryptic species among samples of *Ca. crocodilus*. Additional mitogenomic data from samples of *Ca. crocodilus* and *Ca. yacare* may in the future be beneficial in determining the relationship between these two species.

The relationship between the subgroup [*Crocodylus porosus*, *Cr. siamensis* Schneider, 1801, *Cr. palustris* (Lesson, 1831)] and the subgroup [*Cr. johnstoni* Krefft, 1873, *Cr. mindorensis* Schmidt, 1935, *Cr. novaeguineae*] also needs exploration due to the relative low bootstrap support (Fig. 1). The time-scaled tree shows a consistent topology of this subgroup compared to that of Oaks

Table 3. Node statistics of time-calibrated phylogenetic tree of the Crocodylia. Node numbers (*N*) correspond to Figure 2. Node age posterior means and 95% highest posterior densities (HPD) are from the BEAST analyses. In the previous study, a conservative upper bound of 90 Myr was placed on the root of Crocodylia to extend the likely Campanian origin of Crocodylia (Brochu & Densmore, 2000; Salisbury *et al.*, 2006) by 6.5 Myr (Oaks, 2011)

TMRCA	*N*	Node ages		Oaks, (2011)	
		Mean	95% HPD	Mean	95% HPD
Crocodylia(Crocodylidae+ Gavialidae+Alligatoridae)	1	86.8	83.6–90.02	87.14	81.08–90.00
Alligatoridae	2	53.39	50.77–56.02	65.48	62.53–69.53
Caiman+Melanosuchus+Paleosuchus	3	36.18	34.26–38.16	25.37	20.14–28.81
Alligator	4	33.38	31.33–35.45	47.25	36.83–58.28
Caiman+Melanosuchus	5	17.4	16.36–18.41	12.43	10.31–14.68
Caiman	6	13.81	12.93–14.74	8.34	6.69–9.81
	7	3.58	3.25–3.9	3.28	2.09–4.79
Paleosuchus	8	12.09	11.2–12.94	9.53	7.54–11.31
Crocodylidae+Gavialidae	9	50.13	47.5–52.56	54.53	39.84–63.73
Gavialidae(*Tomistoma+Gavialis*)	10	20.76	19.44–22.13	23.23	16.10–28.97
Crocodylidae(*Osteolaemus+ Mecistops+Crocodylus*)	11	31.41	29.56–32.91	22.1	19.31–25.51
Osteolaemus+Mecistops	12	23.11	21.66–24.5	17.71	15.08–22.15
Crocodylus	13	14.09	13.32–14.82	11.3	8.28–13.61
	14	12.37	11.46–13.23	10.06	7.17–12.41
	15	5.87	5.37–6.38	4.53	2.63–6.81
	16	13.47	12.7–14.16	10.21	7.80–12.32
	17	12.03	11.31–12.77	8.82	6.52–10.60
	18	9.25	8.6–9.94	5.95	4.19–7.94
	19	8.28	7.71–8.85	6.82	5.28–8.32
	20	6.47	6.04–6.89	5.21	4.05–6.31
	21	5.59	5.23–6.02	4.08	2.98–5.31
	22	5.15	4.77–5.53	3.42	2.49–4.37
	23	1.21	1.03–1.41	1.79	1.06–2.57

(2011), indicating the effectiveness of our method for resolving the relationships within Crocodylia.

As stated in the introduction, there have been other studies that have challenged the classical Crocodylia taxonomy with 23 species (e.g. Brochu, 2007; Eaton *et al.*, 2009; Hekkala *et al.*, 2011; Shirley *et al.*, 2014, 2018; Bloor *et al.*, 2015; Muniz *et al.*, 2018; Bittencourt *et al.*, 2019; Murray *et al.*, 2019). Due to limited data of controversial groups in our study (e.g. *Crocodylus*, *Mecistops*, *Osteolaemus* and *Paleosuchus*), we cannot provide more effective results to resolve such problems. On the other hand, to ensure the accuracy of our tree topology, we conducted our phylogenetic analyses with ten outgroup species following the study of Green *et al.* (2014). Our tree topology results from ML and BI analyses show a certain degree of inconsistency (Fig. 1; Supporting Information, Fig. S1). For the ML tree, it is identical to Green *et al.* (2014), in which the Crocodylia and birds form a clade, but the tree topology inferred from BI is inconsistent with the traditional concept in which Crocodylia and turtles form a clade. This phenomenon observed from our phylogenetic trees suggests that these taxa may have different evolutionary patterns. Some specificities of the mitogenome, such as its maternal heritability and its inability to recombine (no crossing-over), may also act as other underlying factors (Boore, 1999). Consistent with our phylogenetic results, previous research (Green *et al.*, 2014) also showed these two tree topologies, although they got different proportions in the statistical tree diagrams, hinting to future research direction.

TEMPORAL AND SPATIAL HISTORY IN CROCODYLIA

The estimated age of the most recent common ancestor (TMRCA) of modern Crocodylia, including all three clades (A, Crocodylidae; B, Gavialidae; C, Alligatoridae) is estimated to be around 86.75 Mya (95% HPD interval 83.55–90.01 Mya) during the Upper Cretaceous period, which is close to the divergence time estimated in several earlier analyses (Oaks, 2011; Green *et al.*, 2014; Nicolaï & Matzke, 2019). The TMRCA of Alligatoridae is 53.33 Mya (95% HPD, 50.59–55.77 Mya) and that of Crocodylidae and Gavialidae is 50.13 Mya (95% HPD, 47.73–52.66 Mya) (Fig. 2), which may be closely related to the changing climate during the Late Palaeocene and Early Eocene (Zachos *et al*., 2001, 2008). The TMRCA of Alligatoridae is 53.39 Mya in our study, later than that of Oaks (2011; approx. 65.5 Mya) and slightly earlier than Shirley *et al.* (2014; approx. 50.5 Mya). As to the TMRCA of Crocodylidae and Gavialidae in this study, it was slightly later than that of previous studies (approx. 54 Mya) (Oaks, 2011; Srikulnath *et al.*, 2015).

Our study also provides the diversification rate of Crocodylia, which began to increase from the late Eocene (about 36 Mya) and shows two diversification peak periods (0–10 Mya and 10–20 Mya; Fig. 3; Supporting Information, Fig. S4), which is roughly consistent with the estimated crocodylian species richness through time in the study of Solórzano *et al.* (2019). According to the fossil record of crocodylians, it shows that this group had a rich species diversity in the past (Solórzano *et al.*, 2019). With time, this species richness underwent long-term reduction and then formed its current relatively low diversity of extant Crocodylia (Solórzano *et al.*, 2019). This study showed that within crocodylians the biodiversity decreases are closely related to environmental changes during the Cenozoic (e.g. decreasing temperatures in northern temperate regions and increasing aridification at lower latitudes; Mannion *et al.*, 2015). Combining these two clues, it may imply that the time-calibrated phylogeny of Crocodylia inferred from the complete mitogenomes could hint at true evolutionary relationships within this order. Most speciation of extant Crocodylia occurred from 25 Mya to the present (Fig. 3; Supporting Information, Fig. S4), suggesting that climate fluctuation of that time played a decisive role in the speciation (Zachos *et al*., 2001, 2008).

Our study provides a time-scaled phylogenetic tree based on mitogenomes, which provides explanations for the evolution of extant Crocodylia. However, future studies needs to further integrate additional evidence from palaeontology, palaeoclimatology and additional molecular studies (Scheyer *et al.*, 2013; Mannion *et al.*, 2015; Groh *et al.*, 2020).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Bayesian phylogenetic tree based on the resulting complete mitochondrial genome sequences (except control region) for all known Crocodylia species.
Figure S2. Alignment of the assemble mitochondrial genome with published same species' mitogenomes within Crocodylidae.
Figure S3. Alignment of the assemble mitochondrial genome with published same species' or closely related species' mitogenomes within Gavialidae and Alligatoridae.
Figure S4. Cumulative curve of diversification rate per million years. The dashed line represents the period of rapid diversification in Crocodylia.