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The Complete Mitochondrial Genome of the Brazilian Cownose Ray *Rhinoptera brasiliensis* (Myliobatiformes, Rhinopteridae) in the Western Atlantic: Genome Characterization and Phylogenetic Analysis

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THE COMPLETE MITOCHONDRIAL GENOME OF THE BRAZILIAN COWNOSE RAY *Rhinoptera brasiliensis* (Myliobatiformes, Rhinopteridae) IN THE WESTERN ATLANTIC: GENOME CHARACTERIZATION AND PHYLOGENETIC ANALYSIS

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Abstract

The Brazilian cownose ray, *Rhinoptera brasiliensis* has undergone a global population reduction and is currently classified by IUCN as Vulnerable. We sequenced the complete mitochondrial genome of *R. brasiliensis*, resulting in a 17,759 bp genome size, featuring the typical vertebrate mitochondrial genome structure. The mtDNA genome contains 13 protein-coding genes (PCGs), two ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes, and a non-coding control region (D-loop). Each PCG was initiated by an authoritative ATG codon, except for COX1 that initiated by a GTG codon. Most of the PCGs were terminated by a complete codon (TAA/TAG), while an incomplete termination codon (TA/T) was found in five out of the 13 PCGs. Both Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were used to reconstruct the phylogenetic relationships which revealed that *R. brasiliensis* was closely related to *R. steindachneri* whereas the reported mitogenome from a single specimen identified as *R. steindachneri* from Sri Lanka (GenBank accession number KM364982) was found to correspond to the sequence for *R. javanica*. These novel results provide a useful resource for further phylogeographic and population genetics studies of *Rhinoptera* spp.

Keywords

Mitogenome, cownose ray, elasmobranch, protein-coding genes, phylogeny

Introduction

The genus *Rhinoptera* is composed of eight morphologically similar species of cownose rays (Rhinopteridae) (Last et al. 2016). Currently, the species of the genus *Rhinoptera* are economically important and at risk due to its late maturity, long gestation, and low fertility (typically one pup each year (Neer and Thompson 2005; Fisher et al. 2013; Burgos-Vázquez et al. 2018) with exceptional reports of broods consisting of 2 embryos (Fisher et al. 2014, Poulakis 2013). Cownose rays have being included in the IUCN Red List of Threatened species: Lusitanian cownose ray *Rhinoptera marginata* (Geoffroy Saint-Hilaire, 1817) as Critically Endangered, flapnose ray *Rhinoptera javanica* Müller and Henle, 1841 and Oman cownose ray *Rhinoptera jayakari* Boulenger, 1895 as Endangered, Pacific cownose ray *Rhinoptera steindachneri* Evermann y Jenkins, 1891 as Near Threatened, Australian cownose ray *Rhinoptera neglecta* Ogilby, 1912 as Data Deficient, cownose ray *Rhinoptera bonasus* (Mitchill, 1815) and Brazilian cownose ray *Rhinoptera brasiliensis* Müller, 1836 as Vulnerable.

The Brazilian cownose ray is a coastal pelagic species that typically migrates in large schools throughout its range. *R. brasiliensis*, had been considered to be endemic to Brazilian coastal waters between Rio de Janeiro and Rio Grande do Sul (Menni and Stehmann 2000; Barker 2006). However, studies using morphological and molecular methods have expanded the range of *R. brasiliensis* to the southern Gulf of Mexico (Palacios-Barreto et al. 2017), the northern Gulf of Mexico (Jones et al. 2017), and more recently the western North Atlantic (Weber et al. 2021). This increased range of distribution in *R. brasiliensis* revealed an overlap with the range of *R. bonasus*, and given the absence of clear external diagnostic characteristics to distinguish between both *Rhinoptera* species, the range of *R. brasiliensis* remains uncertain.

The mitochondrial genome of elasmobranchs is a circular molecule ranging in size from ~16–20 kb, and like most vertebrate mitogenomes is generally encoded for 37 genes composed of 13 protein-coding genes (PCGs), two ribosomal RNAs genes (12S rRNA gene and 16S rRNA gene), 22 transfer RNA (tRNA) genes, and a major non-coding region that contains the initial sites for mtDNA replication, RNA transcription, and an A + T-rich region (Kousteni et al. 2021). Until now, the complete mitogenomes of approximately 300 elasmobranch species have been sequenced, with roughly 30 mitogenomes for Myliobatiformes species, and only one species for the family Rhinopteridae. The first complete mitochondrial genome for *Rhinoptera* was published by Poortvliet et al. (2014) based on an individual collected in Negombo, Sri Lanka (GenBank accession number KM364982), and identified as *R. steindachneri*. Since then, two other partial mitogenomes for *R. bonasus* (GenBank accession number KX151652, White et al. 2018) and *R. steindachneri* (GenBank accession number JN184076, Aschliman et al. 2012), have been reported.

This study provides the first report for the complete mitochondrial genome of the Brazilian cownose ray (*Rhinoptera brasiliensis*) based on specimens from the southern Gulf of Mexico. These new results provide insights into genomic structure, including genome organization and composition, gene content, functional regions, and evolutionary dynamics. Also, using a strategy described by Sangster and Luksenburg (2020) and recently demonstrated with an elasmobranch by Sangster and Luksenburg (2021), we show that the accession KM364982, reported as *R. steindachneri*, belongs to the mitogenome sequence of *R. javanica*. These completely sequenced mitochondrial genomes will provide a broad and useful molecular resource to facilitate future phylogenetic studies to better understand the evolutionary history for this controversial genus.

Materials and methods

Sample collection

We obtained tissue samples from specimens collected as bycatch by artisanal fisheries in the Southern Gulf of Mexico, Seybaplaya, Campeche. The specimens were identified as *R. brasiliensis* based on tooth series counts (McEachran and de Carvalho 2002). A total of four jaws of individuals from localities in Veracruz, Campeche and Quintana Roo, were collected and deposited in the National Fish Collection of the Instituto de Biología, Universidad Nacional Autónoma de México (CNPE–IBUNAM; catalog numbers: CNPE–IBUNAM 22282, 22283, 22284 and 22285). Tissue samples from each specimen were preserved in 95% ethanol for molecular analyses.

DNA extraction and sequencing

Whole-genome DNA (gDNA) was isolated using a standard phenol:chloroform protocol and 200 mg of fin tissue. The DNA was resuspended in 50 µL TE buffer 1X (pH 8) and stored at 4 °C for posterior procedures. For library preparation the isolated gDNA was sheared by sonication with a Bioruptor® using five cycles of sonication of 30 s and 30 s without sonication, to obtain optimal fragments size (≤ 500 bp). The KAPA BIOSYSTEMS® Hyper Prep Kit (KR0961—v4.15, Roche Molecular Systems, Inc, IN USA) was used for library preparation. After end reparation and A-tailing, the DNA was ligated to stubs and libraries were amplified through PCR using Illumina universal iTru primers containing custom nucleotide indexes for both reads (Glenn et al. 2019). Amplified fragments were purified and size selected in a ~ 250–450 pb range with the use of SpeedBeads (Rohland and Reich 2012). This library was normalized and pooled with other iTru libraries from other projects. Library sequencing was carried in an Illumina™ HiSeq X to produce paired-end 150 nucleotide reads at the Georgia Genomics Facility, University of Georgia, Athens, USA.

Genome annotation and sequence analysis

Quality of raw data was assessed with FastQC (Andrews 2010). Good-quality sequences that did not contain ambiguous nucleotides and reads with average quality of > 30 were demultiplexed, trimmed and merged using Geneious Prime 2020.0.4 (https://www.geneious.com). Mitogenome assembly was conducted with MITObim v1.9 (Hahn et al. 2013) using as reference the complete mitochondrial genome of an *R. steindachneri* individual from Sri Lanka (GenBank accession KM364982, Poortvliet et al. 2015) and partial mtDNA genome of *R. bonasus* (GenBank accession KX151652, White et al. 2018). These mitogenomes obtained with the two different assembly strategies were aligned in order to generate a consensus sequence to be used during the annotation procedure. The assembled mitogenome sequence was subsequently annotated using MitoAnnotator on the MitoFish pipeline (http://mitofish.aori.u-tokyo.ac.jp/annotation/input.html; Iwasaki et al. 2013). The resultant annotated *R. brasiliensis* mitochondrial genome was deposited in the GenBank database under accession number OM687170. The nucleotide composition and A+T and G+C contents (%) were calculated for the complete mitogenome and for each of the 13 PCGs for *R. brasiliensis* and the other three mtDNA available for *Rhinoptera*, as well as for the Devil fish *Mobula mobular* (KT203434), the spinetail mobula *Mobula japonica* (NC_018784), and the smoothtail mobula *Mobula thurstoni* (NC_037219). Also, for *R. brasiliensis* codon usage profiles of protein-coding genes (PCGs) were obtained. Relative Synonymous Codon Usage (RSCU) was analyzed using MEGA 11 (Tamura et al. 2021). Composition skew analysis was carried out for *Rhinoptera* and *Mobula* species with the formula AT-skew = (A − T)/(A + T), and GC-skew = (G − C)/(G + C), respectively (Perna and

Kocher 1995). The tRNA genes were identified using the Online Program tRNAscan-SE v2.0 (http://lowelab.ucsc.edu/tRNAscan-SE/; Lowe and Chan 2016) and MITOS WebServer (Bernt et al. 2013). Additionally, the control region was inspected by the program "Tandem Repeats Finder" (https://tandem.bu.edu/trf/trf.html; Benson 1999**).**

Phylogenetic analysis

Phylogenetic analyses were performed with a total of 16 complete or partial mitogenomes of Myliobatiformes downloaded from GenBank including our *Rhinoptera brasiliensis* mitogenome, representing 16 species, five genera, and five families, we used the Brazilian electric ray *Narcine brasiliensis* as outgroup (Supplemental Material, Table S1). Phylogenetic analyses were performed with nucleotide sequences using the 13 concatenated PCGs. DNA sequence data from each PCG were independently aligned via multiple sequence alignment using the software MUSCLE v3.5 (Edgar 2004). The GTR + I + G model was selected as the most likely for sequence evolution using the corrected Akaike Information Criterion ($-\ln L$ = 70026.44, AICc = 140137.19) implemented in JModelTest v2.1.10 (Darriba et al. 2012). Phylogenetic analysis was conducted using maximum likelihood (ML) and Bayesian inference (BI), which were conducted in MEGA 11 (Tamura et al. 2021) and MrBayes 3.2.6 (Ronquist et al. 2012), respectively. The ML method was used to infer phylogenetic trees with 1,000 bootstrap replicates. BI analyses were conducted under the following conditions: 1,000,000 generations, four chains (one cold chain and three hot chains), and a burn-in step for the first 10,000 generations. The confidence values of the BI tree were expressed as Bayesian posterior probabilities. Additionally, to verify the identity of *R. steindachneri* from Sri Lanka (Accession KM364982, Poortvliet et al. 2015) a phylogenetic analysis using sequences of *Rhinoptera* species was obtained using two protein-coding genes (PCGs): NADH dehydrogenase subunit 2 (ND2, 986 bp6quence) (15 sequences), and cytochrome oxidase subunit I (COI, 614 bp; n=24 sequences), including that from *R. brasiliesis* from this study, representing six *Rhinoptera species*). These are among the most commonly used mitochondrial markers in systematic studies of Chondrichthyes and were selected because sufficient numbers of reference sequences were available (Sangster and Luksenburg 2021). For these gene tree analyses, the devil fish *Mobula mobular* (KT203434), the Spinetail mobula *Mobula japonica* (NC_018784), and the smoothtail mobula *Mobula thurstoni* (NC_037219) were used as outgroups (Suppl. Material, Table S1). The HKY+I and GTR+I model was selected as the most likely model of sequence evolution using the corrected Akaike Information Criterion for the COI and ND2 data, respectively.

Results and Discussion

Genome organization and nucleotide composition

In the present study, the complete mitochondrial genome sequence of *Rhinoptera brasiliensis* was determined to be 17,759 bp and was deposited in GenBank (accession OM687170). The mitogenome contained 37 typical mitochondrial genes: 13 vertebrate protein-coding genes, 2 ribosomal RNA (rRNA) genes, 22 tRNAs, and a control region (D-Loop) (Fig. 1; Table 1). Almost every mitochondrial PCGs showed to be encoded on the heavy strand (H strand), excepting the ND6 gene which was found in the L strand (Table 1). The arrangement of these genes was conserved and typical of elasmobranch mitochondrial genomes (Kousteni et al. 2021; Vella and Vella 2021). To date the smallest mitogenome of Myliobatiformes has been reported for the Chilean devil ray *Mobula tarapacana* (15,686 bp) and the largest for the longheaded eagle ray *Aetobatus flagellum* (20,201 bp) (Kousteni et al. 2021). The *R. brasiliensis* mitogenome contained a total of 18 bp overlapping regions between genes, specifically in three pairs of neighboring genes, each ranging from 1 to 9 bp

in length. The longest overlapping region (9 bp) was located between ATP8 and ATP6. A total of 82 bp intergenic spacers (IGS) were dispersed across the mitogenome in 17 positions, ranging from 1 to 35 bp in length (Table 1). The longest IGS was located between $tRNA^{Asn}$ and $tRNA^{Cys}$. These overlapping and intergenic regions are very common in fish mitochondrial genomes (Wang et al. 2020; Sun et al. 2021) and Elasmobranchs (Zhang et al. 2015; Kousteni et al. 2021; Vella and Vella 2021). The nucleotide composition of the *R. brasiliensis* mitogenome was as follows: A (31.1%), T (28.5%), C (27.1%), and G (13.3%), with a slightly bias towards A + T (59.6%) (Table 2). In addition, the A+T contents of PCGs, rRNAs, and tRNAs were also slightly A+T rich (Table 2). The mitochondrial genome strand presents a clear bias in nucleotide composition with a positive AT-skew and a negative GC-skew (AT-skew = 0.043, GC-skew = −0.340), indicating a higher abundance of A over T and of C over G.

Protein-coding genes and codon usage

The 13 PCGs were 11,434 bp in total length, with the longest PCG having 1,839 bp for ND5, while the shortest was 168 bp for ATP8 (Table 1). The average base composition of the 13 PCGs was A (28.0%), T (29.6%), C (26.0%), and G (13.3%) (Table 2). All PCGs initiated with the typical ATG codon with the exception of COX1, which exhibited GTG as starting codon. Eight PCGs (ND1, COX1, COX3, ATPase 8, ND4L, ND5, ND6, and Cytb) terminated with a complete stop codon. The other five PCGs, terminated with an incomplete stop codon TA+ or T++, which seems to be completed as TAA by post-transcriptional polyadenylation at the 3' end of the mRNA (Ojala et al. 1981). These features of the starting and stop codons are also found in other elasmobranchs (Kousteni et al. 2021; Vella and Vella, 2021).

The RSCU values of the 13 PCGs were analyzed and are shown in Suppl. Material, Table S2. The total number of codons, excluding termination codons in the 13 PCGs was 3,811. Among them, CUC (3.54%), CUA (3.52%), and CCU (3.44%) were the most frequent. Likewise, the three most frequent amino acids associated to these PCGs were Leu (15.80%), Ser (9.63%), and Pro (8.37%) (Suppl. Material, Table. S2).

Comparative analysis showed that the nucleotide composition of *R. brasiliensis* is similar to the pattern found in most other Myliobatiformes. The A + T contents of the entire mitogenome, and PCGs are above 50% and the skew statistics reveal a similar pattern of base composition among species. That is, the AT skews ratios for most species were positive and GC were negative, with the exception of *R. steindachneri* (JN184076) for which bot AT and GC skew ratios were negative, although AT larger than GC. The GC skews were all negative (Table 3).

Transfer and ribosomal RNAs

The mitogenome of *R. brasiliensis* contains 22 tRNA genes which produce the typical cloverleaf secondary structure, except for the tRNA-Ser1 which lacks the entire dihydrouridine arm (DHU) (Fig. 2). This unique secondary structure has been commonly observed in many other fishes (Wang et al. 2021; Sun et al. 2021) and elasmobranchs (Kousteni et al. 2021; Vella and Vella, 2021). The 22 tRNA genes have a total length of 1,558 bp, and the length of each tRNA ranges from 68 to 75 bp; $tRNA^{Cys}$ and $tRNA^{Ser}$ were the shortest (68 bp), while $tRNA^{Leu}$ was the longest (75 bp). Of these genes, 14 are encoded on the H strand, and 8 are encoded on the L strand (Table 1). The A + T content of the 22 tRNAs is 62.1%, with a positive AT skew (0.036) and GC skew (0.056) (Table 2).

The two ribosomal genes in the mitogenome, the small subunit of rRNA (12S rRNA) and the large subunit of rRNA (16S rRNA), are encoded on the H-strand. Both genes, were 967 bp and 1,706 bp in length, respectively (Table 1). As in other Chondrichthyes, these two genes are separated by the $tRNA^{Val}$ gene, and located between $tRNA^{Phe}$ and $tRNA^{Leu}$ (Fig. 1, Table 1). The total A + T content of the rRNA genes (59.9%) is lower than that of the total tRNA genes, which is consistent with the content reported for other fishes (Table 2).

Mitochondrial control region

Like in other vertebrates, the mitochondrial control region (CR), is responsible for replication and transcription of the mitogenome (Boore 1999). The control region of *R. brasiliensis* mitochondrial genome, was 2,035 bp in length and is located between $tRNA^{Pro}$ and $tRNA^{Phe}$ genes, as occurs in several elasmobranchs and teleost. This region has a high A + T rich content accounting for 68.4% of total base pairs and has multiple AT-rich tandem repeats along the entire stretch of the CR as detected by Tandem Repeats Finder (Suppl. Material Table. S3). This repetitive motif was also found in other elasmobranchs species, except for longnose spurdog *Squalus blainville* (Kousteni et al. 2021). Moreover, the AT-skew (0.041) has a positive value, and for GC-skew (−0.230) is negative, which means that As and Cs are more abundant than Ts and Gs (Table 2). The high AT content for the CR region shows that the evolution rate of this region is higher than that for other regions of the mitochondrial DNA, so it is suitable for the study of genetic diversity at the species, populations or individual level.

Mitochondrial phylogeny analyses

Based on the concatenated alignment of 13 PCGs of 17 species, the resulting tree for both, BI and ML analyses resulted in quite similar topologies with similar branch lengths and strong support for ML analysis (Bootstrap = 100) and Bayesian inference (Posterior Probability = 1) (Fig. 3). Both analyses recovered a similar arrangement for Myliobatiformes as proposed by previous molecular analysis (Aschliman et al. 2012; Naylor et al. 2012) but in this study included five families. In both analyses, all families formed well-supported monophyletic groups. In both phylogenetic trees, the family Aetobatidae was the sister group to all other Myliobatiformes, and Rhinopteridae is the sister taxon to Mobulidae (Aschliman et al. 2012), and these two are highly supported sisters (0.99) to both Gymnuridae and Pleisiobatidae. This is consistent with previous phylogenetic studies (Kousteni et al. 2021) (Fig. 3). At the species level, phylogenetic reconstruction with available mtDNA genomes suggested that *Rhinoptera brasiliensis* is closely related to *R. steindachneri* and both are a sister group to *R. bonasus*, which was in concordance with a previous morphological study (Jones et al. 2017).

The phylogenetic position of *R. brasiliensis* within the genus *Rhinoptera* was also assessed using the mitochondrial protein-coding gene COI. Both ML and BI phylogenetic analyses supported the division of the Family into four clades. In a gene tree of COI sequences, the *R. brasiliensis* specimens collected in the Gulf of Mexico bear close genetic affinity to those from the Brazilian cownose ray *R. brasiliensis*, previously supported by morphological (Jonas et al. 2017) and molecular studies using the Automatic Barcode Gap Discovery (ABGD) method (Palacios-Barreto et al. 2017). Based on both ML and BI tree topologies, *R. javanica* + *R. neglecta* represent the earliest divergent lineage of the *Rhinoptera* group. Nevertheless, according to the BI mitogenomic phylogeny (Fig. 4a), *Rhinoptera bonasus* was placed at the most basal position being involved in a polytomy with *R. javanica* and the group of *R. jayakari* with *R. marginata* and *R. brasiliensis*. *R. bonasus* was placed

with low ML support (BS = 75%) but strong IB support (PP = 0.99) as the sister group to the *R. steindachneri* + *R. jayakari* group (Fig. 4B).

Additionally, both gene trees showed strong evidence that the reported mitogenome (GenBank accession number KM364982) for *R. steindachneri* is actually an additional sequence for *R. javanica* and not for *R. steindachneri*. The barcoding fragment of the COI gene shows a similarity is 99.66% with respect to *R. javanica*. In contrast, similarity in relation with *R. steindachenri* (GenBank accession number JN184076) was 92.12%, i.e. at least 7% of the interspecific divergence in the cox1 gene. This result is confirmed by the phylogenetic analyses of cox1 – and also ND2 – data sets, both of which consistently place the new sequences within *R. javanica* instead of *R. steindachenri*, exhibiting strong support of both mitochondrial clades (Fig. 4b), and suggesting that there is a specie misidentification in the previous report of Poortvliet et al. (2014) for *R. steindachenri*. In terms of sequences divergence of 13 PCGs of the *R. steindachneri* (KM364982) with respect to (JN184076) was 8.68%. No existing mitogenome of *R. javanica* is available for comparison, but sequence divergence in ND2 between *R. steindachneri* (KM364982) and the others *R. steindachneri* (JQ518918, HQ540561, HQ540559, HQ540560), vary between 9.9 and 13.5%. In contrast, sequence divergence with respect to *R. javanica* (MK335278, KM396924, JQ518924, MG774927, MG774923, MH841991, MG792117, MG792069) was 0.0–0.42% for ND2 and 0.19% for COI, respectively.

These species are strongly allopatric with widespread geographic ranges believed to reflect their high mobility (Schwartz 1990). However, given that *R. javanica* is distributed in the Indian Ocean and South Asia while *R. steindachneri* occurs in the eastern Pacific Ocean and the Gulf of California, these two species have apparently non-overlapping geographic ranges and therefore the occurrence of hybrids seems unlikely. Given that the area where the supposed individual from *R. steindachneri* was captured (Sri Lanka), and the phylogenetic position analyses of the complete mitogenome and the two individual genes (COI and ND2), is an example highlights the common difficulties encountered when identifying Rhinopterids based on morphology alone.

Conclusions

In the present study we sequenced and described for the first time, the complete mitogenome for *R. brasiliensis*, which is 17,759 bp and contains 37 genes and one control region as typically occurs for most vertebrates mitogenomes. The particular features of this newly sequenced mitogenome are mostly consistent with those reported in other elasmobranch mitogenomes. The phylogenetic tree obtained for the complete mitogenome and the individual genes, confirmed the monophyly of Rhinopteridae. Our results also suggest that the mitogenome corresponding to the GenBank accession number KM364982 and originally attributed to *R. steindachneri*, differs in two mitochondrial DNA genes from *R. steindachneri* and is nearly identical to *R. javanica*, which is Critically Endangered. The results of the present study will be useful for further investigation of the evolutionary relationships within Rhinopteridae. To better address the phylogenetic and biogeographical complexities of Rhinopterids, additional species of Rhinopteridae need to be further explored, including more complete mitochondrial genome sequencing.

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Figure 1. Circular map of the mitochondrial genome of *Rhinoptera brasiliensis*. Protein coding and ribosomal genes are shown with standard abbreviations. Coding and non-coding regions are labeled. Two rRNA genes (in light beige); 13 coding genes (in black); 22 tRNA genes (in red) and control region (D-loop) (in brown). The genes in the inner are encoded on the L-strand. (Color figure online)

Figure 2. Putative secondary structures for 22 tRNAs from the mitochondrial genome of *Rhinoptera brasiliensis*. The tRNAs are labeled with the abbreviations of their corresponding amino acids. Dashes indicate Watson–Crick base pairing and dots indicate G–C base paring.

Figure 3. Phylogeny of Myliobatiformes based on the concatenated dataset of 13 mtDNA protein-coding genes (A) Bayesian inference. (B) Maximum Likelihood analyses based on nucleotide data. Posterior probabilities and bootstrap values are presented next to the nodes. Mitogenomes assembled for the first time in this study are indicated by blue label.

Figure 4. Phylogenetic relationships of *Rhinopteridae* based on (A) COI, (B) ND2, using Bayesian inference (left) and Maximum Likelihood (right) analyses. Posterior probabilities and bootstrap values are presented next to the nodes. Note the consistent placement of KM364982 with *Rhinoptera javanica*, red labels.

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Table 1. Mitochondrial genome organization of *Rhinoptera brasiliensis*.

		Position					Codon		
Gene	**Strand[a]**	**From**	**To**	**Length (bp)**	**Intergenic nucleotide (bp)**	**Anticodon**	**Start**	**Stop[c]**	**No AA**
tRNA^{Phe} (F)	H	1	69	69	0	TTC			
12s rRNA	H	70	1036	967	0				
tRNA-Val (V)	H	1037	1108	72	0	GTA			
16s rRNA	H	1109	2814	1706	0				
tRNA-Leu (L)	H	2815	2889	75	0	TTA			
NAD1	H	2891	3865	975	1		ATG	TAA	324
tRNA^{Ile} (I)	H	3867	3936	70	1	ATC			
tRNA^{Gln} (Q)	L	3939	4010	72	2	CAA			
tRNA^{Met} (M)	H	4011	4079	69	0	ATG			
NAD2	H	4080	5125	1046	0		ATG	TA+	348
tRNA^{Trp} (W)	H	5126	5195	70	1	TGA			
tRNA^{Ala} (A)	L	5197	5265	69	1	GCA			
tRNA^{Asn} (N)	L	5267	5339	73	35	AAC			
tRNA^{Cys} (C)	L	5375	5442	68	5	TGC			
tRNA^{Tyr} (Y)	L	5448	5517	70	1	TAC			
COX1	H	5519	7072	1554	8		GTG	TAA	517
tRNA^{Ser} (S)	L	7080	7150	71	0	TCA			
tRNA^{Asp} (D)	H	7151	7221	71	4	GAC			
COX2	H	7225	7915	691	0		ATG	T++	230
tRNA^{Lys} (K)	H	7916	7989	74	1	AAA			
ATP8	H	7991	8158	168	-8		ATG	TAA	55
ATP6	H	8149	8831	683	0		ATG	TA+	227
COX3	H	8832	9617	786	5		ATG	TAA	261
tRNA^{Gly} (G)	H	9623	9694	72	0	GGA			
NAD3	H	9695	10043	349	0		ATG	T++	116
tRNA^{Arg} (R)	H	10044	10114	71	0	CGA			
NAD4L	H	10115	10411	297	-5		ATG	TAA	98
NAD4	H	10405	11785	1381	0		ATG	T++	460
tRNA^{His} (H)	H	11786	11854	69	1	CAC			
tRNA^{Ser} (S)	H	11856	11923	68	2	AGC			
tRNA^{Leu} (L)	H	11926	11997	72	0	CTA			
NAD5	H	11998	13836	1839	-2		ATG	TAA	612
NAD6	L	13833	14354	522	0		ATG	TAG	173
tRNA^{Glu} (E)	L	14355	14423	69	6	GAA			
Cyt b	H	14430	15572	1143	3		ATG	TAA	380
tRNA^{Thr} (T)	H	15576	15648	73	5	ACA			
tRNA-Pro (P)	L	15654	15724	71	0	CCA			
D-loop	H	15725	17759	2035	0				

Notes:

[a] Genes encoded on the heavy strand (H) or Light strand (L).
[b] Negative numbers indicate overlapping nucleotides
[c] TA+ and T++ indicate incomplete stop codons

Table 2. Nucleotide contents of the coding and non-coding regions of the mitochondrial genome of *Rhinoptera brasiliensis*, indicating AT-, GC-skew ratios.

	Length (bp)	A%	T%	G%	C%	A+T%	AT-skew	GC-skew
Mitogenome	17,759	31.1	28.5	13.3	27.1	59.6	0.043	-0.340
PCGs	11,434	28.0	29.6	13.3	29.0	57.6	-0.028	-0.371
1st codon position	3,812	29.1	27.7	13.4	29.8	56.8	0.025	-0.382
2nd codon position	3,811	26.4	33.0	12.7	27.8	59.5	-0.111	-0.374
3rd codon position	3,811	28.5	28.2	14.0	29.4	56.6	0.006	-0.356
rRNA	2,673	34.7	25.3	17.2	22.9	59.9	0.157	-0.143
tRNA	1,558	32.2	29.9	20.0	17.9	62.1	0.036	0.056
D-loop region	2,035	35.6	32.8	12.2	19.5	68.4	0.041	-0.230

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Table 3. Nucleotide contents of genes and the mitochondrial genome skew in different mitogenomes of *Rhinoptera* and *Mobula* species.

		Entire genome								Protein-coding genes			
Species	**GenBank Accession #**	**Lenght (bp)**	**A (%)**	**T (%)**	**C (%)**	**G (%)**	**AT (%)**	**AT skew**	**GC skew**	**Length (bp)**	**AT (%)**	**AT skew**	**GC skew**
Rhinoptera brasiliensis	OM687170	17,759	31.1	28.5	27.1	13.3	59.6	0.043	-0.340	11,434	57.6	-0.028	-0.371
Rhinoptera steindachneri	KM364982	15,480	30.6	28.4	27.6	13.4	58.9	0.036	-0.346	11,196	58.4	-0.039	-0.366
Rhinoptera steindachneri	JN184076	10,865	28.4	29.1	30.0	12.4	57.5	-0.011	-0.041	10,864	57.5	-0.011	-0.414
Rhinoptera bonasus	KX151652	15,707	30.4	28.0	27.9	13.5	58.5	0.041	-0.348	11,434	57.7	-0.033	-0.365
Mubula mobular	KT203434	18,913	32.9	29.7	24.7	12.6	62.6	0.051	-0.323	11,441	59.8	-0.041	-0.355
Mobula japanica	NC_018784	18,880	32.7	29.7	24.7	12.7	62.4	0.047	-0.317	11,423	59.7	-0.044	-0.352
Mobula thurstoni	NC_037219	17,610	30.6	29.1	26.5	13.6	59.7	0.026	-0.319	11,440	58.2	-0.043	-0.348

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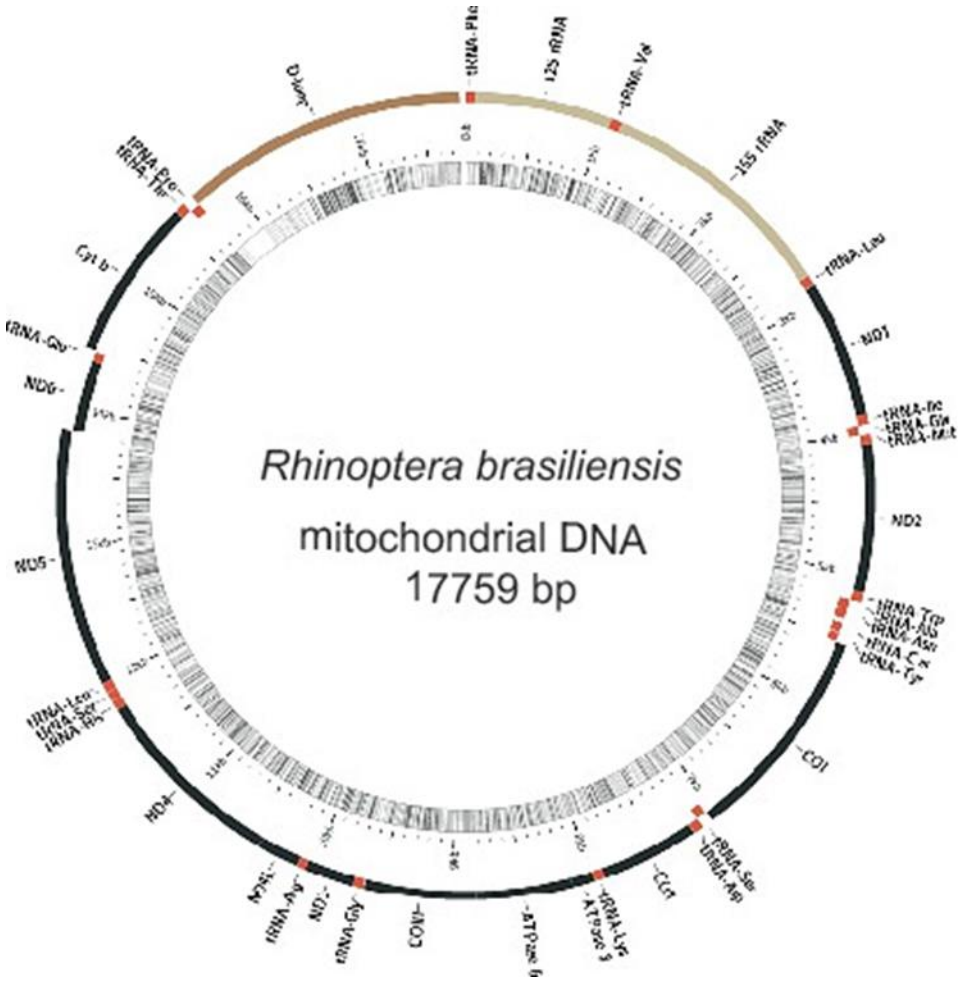

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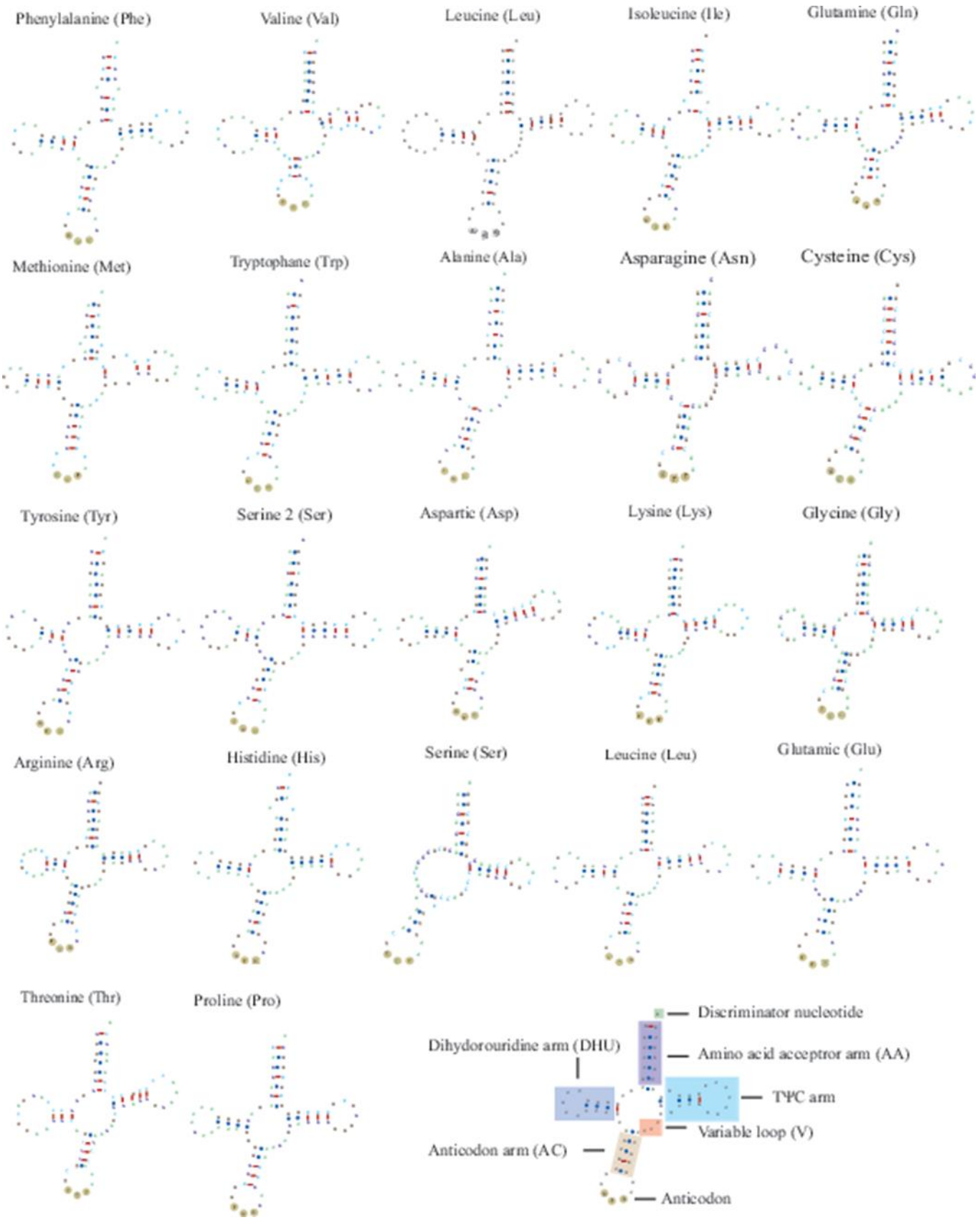

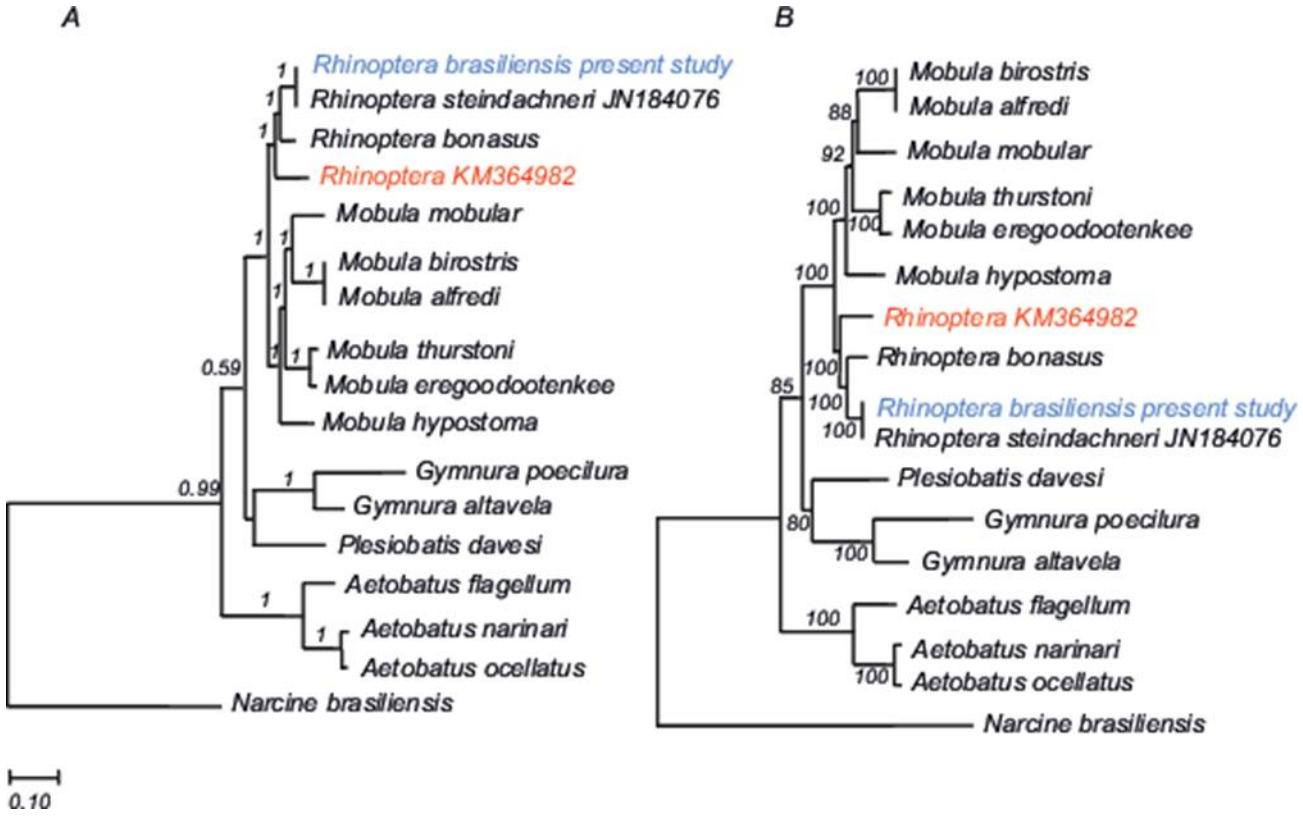
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Mobula hypostoma
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Gymnura altavela
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Aetobatus flagellum
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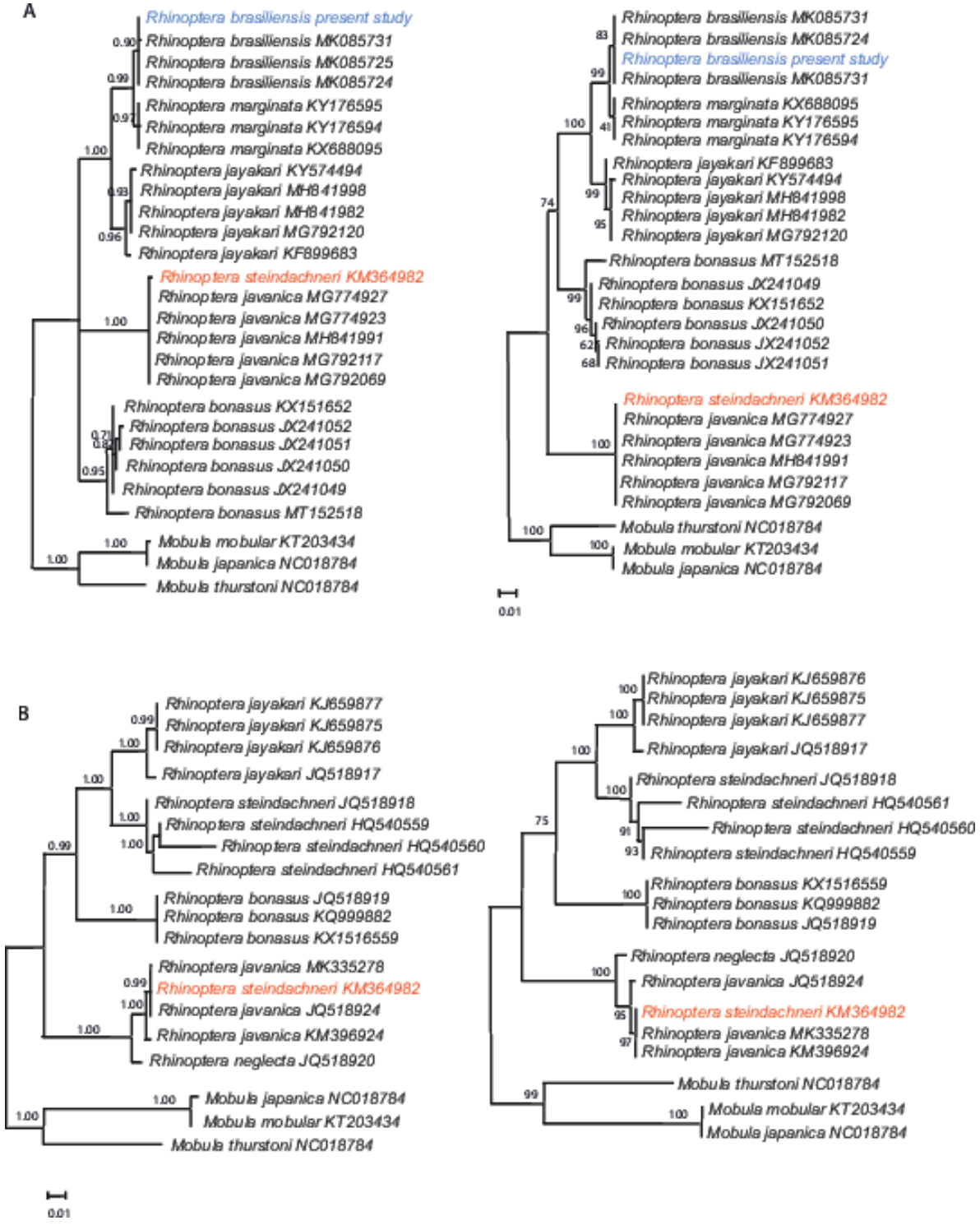
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