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Mitochondrial genome to aid species delimitation and effective conservation of the Sharpnose Guitarfish (*Glaucostegus granulatus*)

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ABSTRACT

We present the complete mitochondrial genome of the Sharpnose/Granulated Guitarfish *Glaucostegus granulatus*, obtained with whole genome shotgun sequencing of genomic DNA. The 16,547 bp long circular genome consisted of 13 protein-coding genes, 22 tRNA genes, 2 rRNA genes, and a non-coding control region. A few protein-coding genes ended with incomplete stop codons. Phylogenetic analysis provided strong support for the specimen to be identified as *G. granulatus* and improved resolution of phylogeny within the genus Glaucostegus such as placing *G. thouin* in a sister group with *G. typus*. This is the first complete mitogenome within the genus Glaucostegus and will be beneficial to future molecular taxonomic studies and species identification, population studies and conservation efforts involving *G. granulatus*.

1. Introduction

The Sharpnose Guitarfish (*Glaucostegus granulatus*) is one of fifteen critically endangered Rhino Rays found in marine neritic and intertidal habitats of the northern Indian ocean, where it ranges from the Gulf of Oman and Persian Gulf to Myanmar (Kyne et al., 2019). This species, alongwith other Rhino Rays has been exploited as incidental catch, and this has led to severe population declines, and several localized disappearances (Dulvy et al., 2017; Moore, 2017; Jabado et al., 2018). Like many chondrichthyan species, there are no species-specific time-series data available for the sharpnose guitarfish that can be used to calculate population reduction (Kyne et al., 2019). This is due to a lack of species-specific reporting as well as limitations in accurate taxonomic identification. As a result current Red List assessments are made from contemporary landings and catch rate datasets from range countries at varying levels of taxonomic resolution (eg. 'Rhinobatids' to 'Guitarfishes' to specific measurements for other sympatric species such as *Glaucostegus halavi* or *G. thouin* or *G. typus* or even Mobulid species). Based on these data, overall declines of > 80% were estimated for the Sharpnose Guitarfish populations throughout its range. However, it is known that, aggregated catches mask overfishing and local extinctions (Dulvy et al., 2000), underpinning the urgent need to enable species-specific reporting for the critically endangered Sharpnose Guitarfish and other Rhino Rays.

Further, Rhino Rays including the Sharpnose Guitarfish were listed on Appendix II of the Convention for International Trade in Endangered Species (CITES), in August 2019 (CITES Going Full Steam Ahead to Ensure Sustainable Use of Marine Resources | CITES, 2019). The resulting CITES trade restrictions obligate nations to monitor and regulate all international exports of the species and require permits for sustainable and legal harvests of the species for exports. Most fisheries takes of the Sharpnose Guitarfish are intense and exploitative, poorly monitored, and unregulated with respect to CITES monitoring and stock assessments (Kyne et al., 2019; Moore, 2017). A crucial aspect of improved monitoring regimes for *G. granulatus* and other Rhino Ray fisheries is to increase capacity for species specific reporting and accurate species identification using molecular taxonomy. Such improvements are vital in order to obtain the most accurate population estimates for each species from landing or catch data, to ensure efficient enforcement of trade laws in determining export quotas for each species

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Fig. 1. Dorsal view of specimen along the entire length (A), zoomed in views of the frontal (B) and caudal portions (C). Specimen identified as *Glaucostegus granulatus* using morphology.

under CITES protections and, last in order to ensure the most efficient protections for remaining populations of the species.

The most commonly used identification methods for Rhino Rays currently rely on morphological identification which requires extensive training and expertise, and in the absence of expert advice, which is often the case, leads to misidentifications. Molecular taxonomic studies of the Sharpnose Guitarfish and Rhino Rays are limited in scope to single genetic markers (Naylor et al., 2012a), which are not always accessible for all species and do not allow higher resolution analyses of phylogenetic and evolutionary processes. In addition limited genetic markers restrict genetic assessments of population structure and biogeography which further affect conservation and management of the species under consideration (Li et al., 2015; Delser et al., 2016; Pazmiño et al., 2018).

We here report the first complete mitochondrial genome of the Sharpnose Guitarfish, which was obtained by genome skimming of genomic DNA obtained from fins slated for export. The mitogenome reported is the first for *G. granulatus* and has enabled us to improve species delimitation as well as phylogenetic relationships of taxa in the genus Glaucostegus.

2. Methods

2.1. Sampling and DNA sequencing

DNA was extracted from a fin clip of an individual female guitarfish specimen (Fig. 1) collected in Veraval, Gujarat, India as outlined in (Johri et al., 2019a). Genomic DNA libraries were prepared using Accel-NGS 2S DNA Library Kit for Illumina platform (Swift Biosciences). The genomic library was sequenced on an Illumina MiSeq (MiSeq Reagent Kit v3). Although the run was designed to be a PE sequencing run, only single reads in the forward direction were obtained due to an error

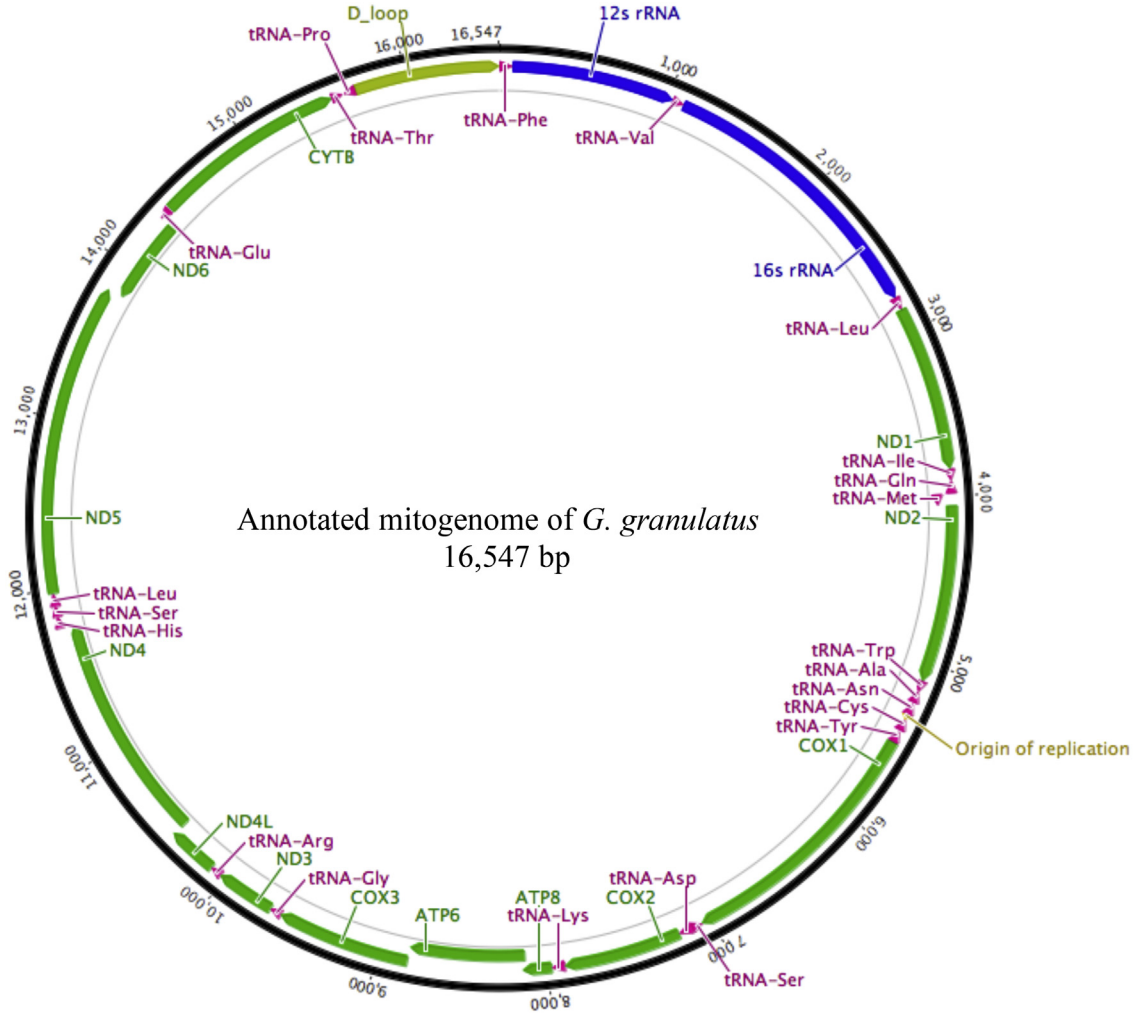

Fig. 2. Annotated mitogenome sequence indicating gene position and topology of each loci, with -protein-coding sequences in black, tRNAs in magenta, rRNA in blue, and the control region in green. Outer circle represents aligned genes with outermost representing the heavy strand and genes on the complementary l light strand depicted on the inside. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

midway through sequencing.

2.2. Sequence analysis

Metagenomic sequence reads were quality filtered using the PReprocessing and INformation of Sequences tool, PRINSEQ (Schmieder and Edwards, 2011) to remove artificial replicate (Gomez-Alvarez et al., 2009), while low quality sequence reads were trimmed to contain < 5 bases of a Q-score ≤15 (Cox et al., 2010). The quality filtered genomic sequence reads were then mapped to a reference mitochondrial sequence (Accession # KF612341.1 *Carcharhinus sorrah*) in the Geneious environment and assembled into a contig as previously described (Johri et al., 2019a). Simultaneously *De novo* contig assembly was conducted with genomic sequences using the SPAdes assembler (Bankevich et al., 2012) on a computing server.

However, an accurate and complete mitochondrial genome was obtained through the Geneious mapping and assembly pipeline as described previously (Johri et al., 2019a). The resulting mitochondiral genome sequence was annotated within Geneious through the alignment and annotation transfer tool by comparison with annotated elasmobranch mitochondrial genomes from GenBank (NCBI Resource Coordinators, 2017). Annotation using MitoAnnotator on the MitoFish website (Iwasaki et al., 2013) was also performed with unsatisfactory results.

Taxonomic identity of the sample was determined through morphological identification and phylogenetic assessment. Morphological identity was established through methods described in Rays of the World (Rays of the World, Peter Last, William White, Marcelo de Carvalho, Bernard Séret, Matthias Stehmann, Gavin Naylor, 9780643109131, 2019) and assessments on iNaturalist, an initative of the California Academy of Sciences and National Geographic (DOI: https://www.inaturalist.org/observations/29997970). To assess phylogenetic placement of the specimen, gene trees were constructed using mitochondrial sequences for Cytochrome oxidase 1 (COX1), and NADH dehydrogenase subunit 2 (NADH2 or ND2) obtained from GenBank (NCBI Resource Coordinators, 2017) (Table S1). The genus Glaucostegus is not fully represented in any gene currently available on GenBank (five species each available for COX1, and NADH2). Genes were therefore analyzed separately and then in a concatenated matrix to assess congruence between phylogenies. Due to a large amount of missing data for the full COX1 gene, all COX1 sequences were trimmed to 717 bp, the smallest available sequence. Multiple samples from *G. granulatus* and *G. thouin* were included to address monophyly of these two taxa since early analyses rendered them paraphyletic. All sequences were aligned using MUSCLE 3.8.31 (Edgar, 2004) and PartitionFinder 2.1.1 (Lanfear et al., 2016) was used to identify optimal partitioning schemes and the best-fit model of molecular evolution, which was then used for all downstream analyses. In order to ensure robustness of the phylogenetic estimates, phylogenies were inferred in both Maximum Likelihood (IQ-Tree v1.6.10) (Nguyen et al., 2015) and Bayesian

Table 1
Annotation table of genes identified in the *G. granulatus* mitochondrial sequence with their start and end positions on the forward or reverse strands.

Name	Type	Minimum	Maximum	Length	Direction
tRNA-Phe	tRNA	3	73	71	Forward
12s rRNA	rRNA	74	1035	962	Forward
tRNA-Val	tRNA	1036	1107	72	Forward
16s rRNA	rRNA	1108	2801	1694	Forward
tRNA-Leu	tRNA	2802	2876	75	Forward
ND1	gene	2878	3850	973	Forward
tRNA-Ile	tRNA	3851	3920	70	Forward
tRNA-Gln	tRNA	3921	3992	72	Reverse
tRNA-Met	tRNA	3992	4062	71	Forward
ND2	gene	4063	5107	1045	Forward
tRNA-Trp	tRNA	5109	5178	70	Forward
tRNA-Ala	tRNA	5180	5248	69	Reverse
tRNA-Asn	tRNA	5250	5323	74	Reverse
Origin of replication	origin_of_replication	5324	5358	35	Forward
tRNA-Cys	tRNA	5359	5426	68	Reverse
tRNA-Tyr	tRNA	5429	5497	69	Reverse
COX1	gene	5499	7055	1557	Forward
tRNA-Ser	tRNA	7059	7128	70	Reverse
tRNA-Asp	tRNA	7130	7200	71	Forward
COX2	gene	7208	7898	691	Forward
tRNA-Lys	tRNA	7899	7972	74	Forward
ATP8	gene	7974	8141	168	Forward
ATP6	gene	8132	8815	684	Forward
COX3	gene	8820	9605	786	Forward
tRNA-Gly	tRNA	9607	9676	70	Forward
ND3	gene	9677	10,027	351	Forward
tRNA-Arg	tRNA	10,026	10,097	72	Forward
ND4L	gene	10,098	10,394	297	Forward
ND4	gene	10,388	11,768	1381	Forward
tRNA-His	tRNA	11,769	11,837	69	Forward
tRNA-Ser	tRNA	11,838	11,903	66	Forward
tRNA-Leu	tRNA	11,904	11,975	72	Forward
ND5	gene	11,976	13,817	1842	Forward
ND6	gene	13,813	14,331	519	Reverse
tRNA-Glu	tRNA	14,333	14,401	69	Reverse
CYTB	gene	14,405	15,547	1143	Forward
tRNA-Thr	tRNA	15,552	15,624	73	Forward
tRNA-Pro	tRNA	15,627	15,696	70	Reverse
D_loop	D_loop	15,698	16,547	850	Forward

Inference frameworks, MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012). For the IQ-Tree analyses, 100,000 ultrafast bootstrap replicates (Wang et al., 2018) were generated, beginning from 100 starting trees. MrBayes phylogenetic inference were run described in (Johri et al., 2019a). All analyses were run on XSEDE on CIPRES Scientific Gateway (Miller et al., 2010).

3. Results and discussion

The mitochondrial genome of *G. granulatus* (GenBank Accession# MN783017) is 16,547 bp in length (Fig. 2) and consists of 13 protein-coding genes (PCGs), 22 tRNA genes, 2 rRNA genes, and a non-coding control region (D-loop) (Table 1). The GC content is 40.0% and the control region is 849 bp long.

Phylogenetic analyses of COX1 sequences placed the specimen within *Glaucostegus granulatus* with statistically significant support, however, much of the remainder of the tree has poor support in both the MrBayes and IQ-Tree analyses of COX1 due to poor data availability for wedgefishes. These analyses place *G. thouin* within *G. granulatus* with strong support, rendering *G. granulatus* paraphyletic at this marker.

Our analyses of NADH2 eliminated the possibility of any resemblance of the specimen to *G. halavi*, which was not present in the COX1 dataset due to sequence unavailability. ND2 runs also recovered poor support throughout the remaining phylogenetic tree using both MrBayes and IQ-Tree analyses.

Phylogenetic analyses of the concatenated matrix again placed the specimen within *G. granulatus*, and rendered *G. granulatus* paraphyletic with strong support. While MrBayes recovered high support throughout the in-group taxa (Fig. 3A), IQ-Tree analysis recovered moderate bootstrap support throughout the remainder of the tree (Fig. 3B). Furthermore, our MrBayes analysis placed one *G. thouin* sample as sister to *G. typus* rather than in the *G. granulatus* + *G. thouin* clade (Fig. 3A).

We present the most extensively-sampled published phylogeny of the genus Glaucostegus by including six species, two of which were represented by multiple samples. Our phylogenetic analyses confidently place the specimen under consideration within *G. granulatus*, matching its taxonomic classification to *G. granulatus* using morphological parameters. While our analyses support the monophyly of Glaucostegus and broadly find similar relationships as previous work (Aschliman, 2011; Naylor et al., 2012b), resolution of the full phylogenetic tree is limited by lack of available data. Full resolution of relationships among the Rhinobatid species will require additional genetic or genomic datasets and inclusion of taxa which currently lack sequence data (*G. microphthalamus* and *G. spinosus*).

In addition to phylogenetic placement of our specimen, our analyses places two previously reported samples of *G. thouin* within *G. granulatus* and one other as sister to *G. typus* as also reported by Aschliman, N. 2011 (Aschliman, 2011), suggesting undescribed diversity or complex demographic processes within Glaucostegus. Further sampling of species within this genus with additional markers will be required to resolve these relationships.

The current report presents the first complete mitochondrial genome of *G. granulatus* (GenBank Accession# MN783017). These genomic data will significantly aid assessment of conservation status and assist implementation of trade regulations for the species through

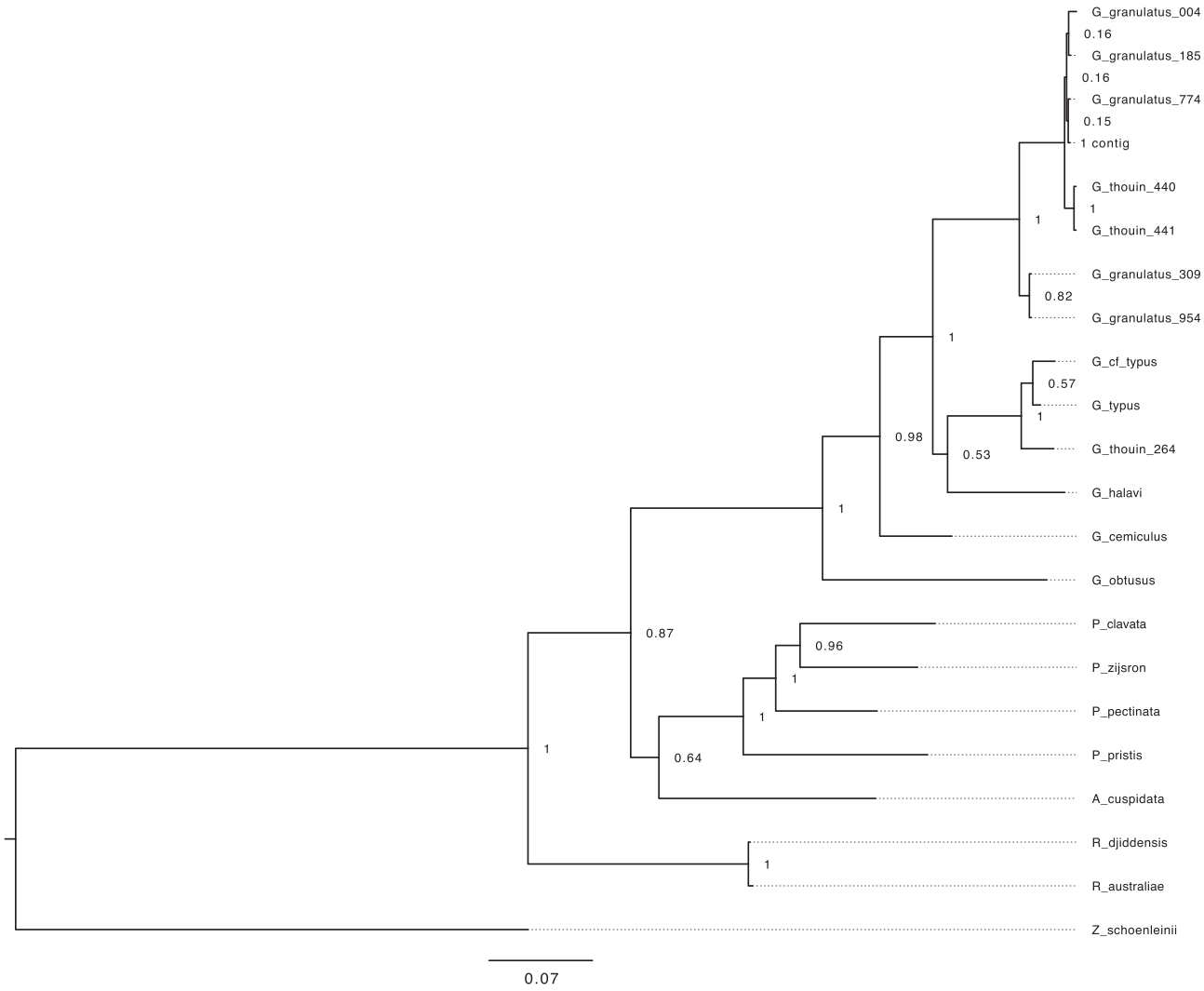

Fig. 3A. Bayesian estimate of relationships among representative taxa from orders Rhynchobatiformes, and Pristiformes. Bayesian phylogenetic estimates determined from concatenated sequences of COI and NADH2 mitochondrial genes from 21 species with *Zanobatos schoenleini* as outgroup. The unknown sample clusters with *G. granulatus*. Numbers at nodes are posterior probabilities.

improved species identification and population genetic assessments. Overall, the genomic sequences and the analyses presented in this report are a step forward in reducing the data deficiency of the species and for the genus Glaucostegus.

Identification of species alone is not sufficient to combat the complicated international trade networks involved in illegal import-export of shark and ray products. International fin shipments change several hands and shipping containers during which they are also repackaged and relabeled, potentially allowing several opportunities to mix illegally harvested fins (and other products) with legal harvests (Mustain et al., 2016). For example a species of shark may be protected in one country but not in another country, and the inability to differentiate between stocks from the two nations could allow laundering of illegally fished populations of the species (unpublished data from OCEANSASIA). Indeed illegal global trading of endangered and CITES listed species has been reported by several studies. Thus, for efficient enforcement of species protections it is critical to differentiate between different species and stocks of protected or CITES listed species such as the sharpnose guitarfish, to ensure that the fins are not being sourced illegally from protected populations. One of the main factors limiting species and stock identification is the enormous gap in genetic data on Chondrichthyes. Very few of the approximately 1200 Chondrichthyes have been assessed with respect to their population genetics (Dudgeon et al., 2012; Johri et al., 2019b), and as a result it remains challenging to differentiate species as well as intraspecific populations of a species. The mitochondrial genome presented here will potentially enable future studies on population genetics of the sharpnose guitarfish using nuclear SNP analyses and multiple mitochondrial markers.

Author contributions

SJ contributed to concept, sampling, sequencing, bioinformatics and wrote the manuscript, JS contributed to sampling, DNA extractions and sequencing, MMora, IL, AG and AB contributed to sequence analyses, ED contributed to editing of the manuscript, SF did the phylogenetic analyses, AT contributed to sampling, and community networks to understand fisheries practices, AC and RE assisted with bioinformatics, MMorris and MD assisted with sequencing.

Data availability statement

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

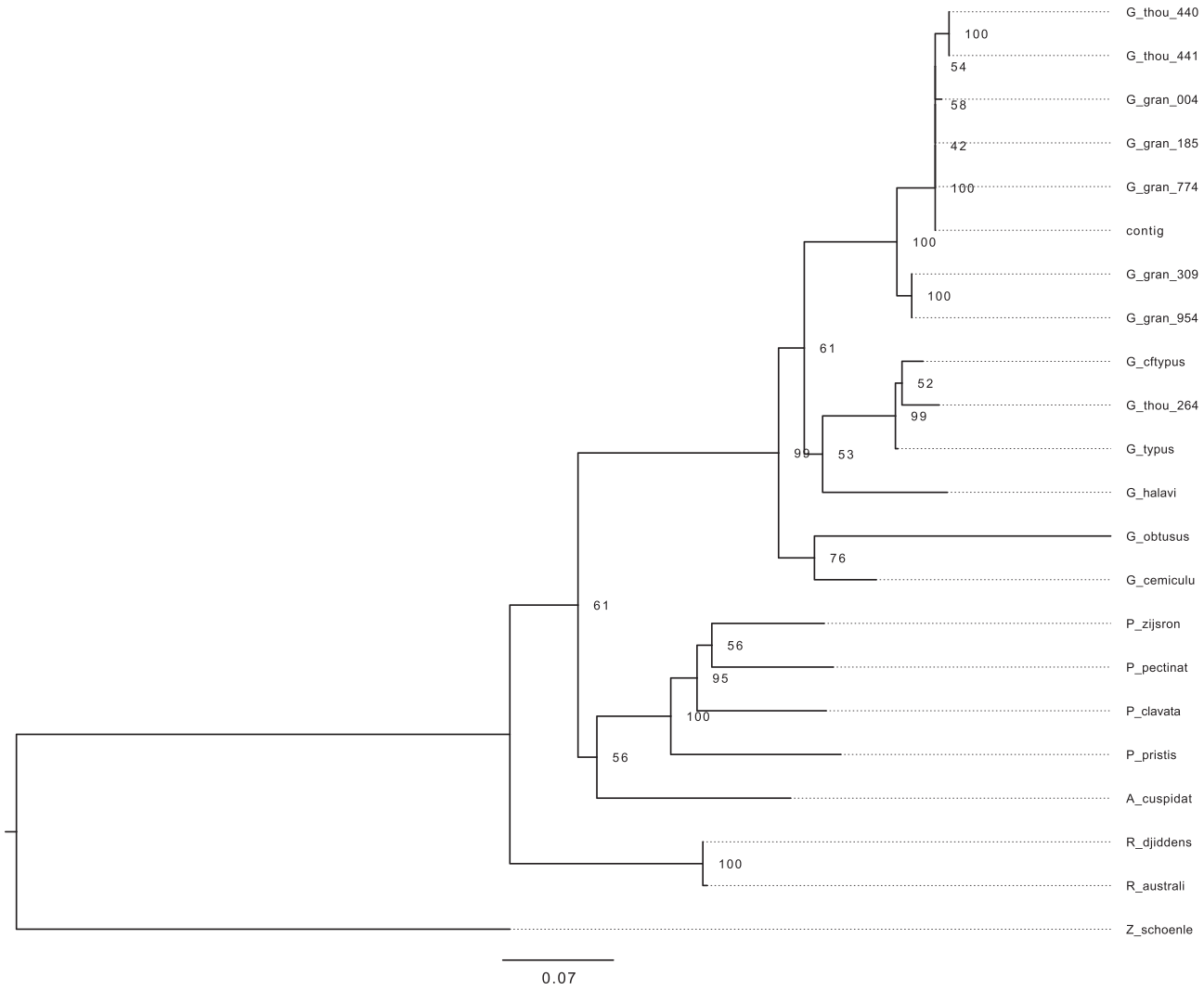

Fig. 3B. Maximum-likelihood estimate of relationships among Rhyncobatiformes and Pristiformes using concatenated mitochondrial protein coding sequences. Maximum-likelihood phylogenetic estimates determined from concatenated sequences of COI and NADH2 mitochondrial genes from 21 species with *Zanobatos schoenleini* as outgroup. The unknown sample clusters with *G. granulatu*. Numbers at nodes are bootstrap support values

Declaration of Competing Interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mgene.2020.100648.

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