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Original research article

Geographical variation in mitogenomes of the largetooth sawfish *Pristis pristis*: Challenges and perspectives for conservation efforts

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ABSTRACT

Sawfishes (Pristidae) have been severely impacted by coastal development and unregulated fisheries and are considered Critically Endangered by the IUCN Red List. Environmental DNA (eDNA) analyses have shown potential for monitoring elasmobranch species, with various studies focusing on using species-specific approaches to detect *Pristis* species. However positive detection using existing probes has not been confirmed in some geographic regions where they would be expected. Here, we aimed to verify the phylogenetic relationships within the Pristidae family, with a particular focus on *P. pristis* (Linnaeus, 1758) to test whether mutations at key sites have been detrimental to species-specific detection of *P. pristis* using the existing probe set. To test this hypothesis mitogenomes were assembled that were found to follow the typical pattern of vertebrate mitogenomic organization. Phylogenetic trees showed similar topologies and confirmed

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geographic mitochondrial variation in *P. pristis*. Mismatches for the published 12S species-specific probe set for *P. pristis* were identified that prevent amplification of positive control samples from Brazil. However, ddPCR detection of the positive control was possible using a newly designed species-specific probe set. This study highlights how geographical variation can severely impact the success of generally applying species-specific detection systems developed based on data from only one geographical region. The new mitogenomes and species-specific probe set developed here may also contribute to improving the potential to map and monitor these Critically Endangered species across the globe.

1. Introduction

Sawfishes (Pristidae) were once common in soft-bottom habitats of shallow, warm waters worldwide, often holding significant cultural importance as reflected in mythology and folklore (Robillard and Séret, 2006; Harrison and Dulvy, 2014; McDavitt, 2014; Moore, 2017; Cabanillas-Torpoco et al., 2023). However, over the past three decades, their populations have been severely impacted by coastal development and unregulated fisheries (Cavanagh et al., 2005; Dulvy et al., 2014). Today, sawfishes have largely disappeared from vast areas at local, regional, and global scales mainly caused he are illegally fisheries or taken as bycatch (Fernandez-Carvalho et al., 2014; Moore, 2015; Dulvy et al., 2016; Leeney and Downing, 2016). They are now considered the most threatened family of elasmobranchs globally (Dulvy et al., 2014), comprising five extant species: *Anoxypristis cuspidata* (Latham, 1794), *Pristis pristis* (Linnaeus, 1758), *Pristis pectinata* Latham, 1794, *Pristis zijsron* Bleeker, (1851), and *Pristis clavata* Garman, (1906) (Faria et al., 2013). All are currently listed as "Critically Endangered" on the IUCN Red List (Carlson et al., 2022; Espinoza et al., 2022; Grant et al., 2022; Harry et al., 2022; Haque et al., 2022).

The application of molecular methods in recent years has significantly advanced the study of elasmobranchs, leading to the description of new species (Naylor et al., 2012, White et al., 2013), the reorganization and resurrection of families and the reassignment of taxa (Last et al., 2016; White and Naylor, 2016; Rodrigues-Filho et al., 2023). These methods have also revealed the presence of cryptic species (Sales et al., 2019; Gales et al., 2024; Petean et al., 2024). In sawfishes, morphological and molecular data have clarified phylogenetic relationships (Faria et al., 2013), and molecular studies have revealed population structure in *P. pristis* from Australian waters (Phillips et al., 2011; Feutry et al., 2015).

Among sawfishes, the Largetooth Sawfish (*P. pristis*) is one of the most iconic species and serves as the type species of its genus (Bigelow and Schroeder, 1953). Originally described by Linnaeus (1758) as having a distribution "in Europa", the species is now considered to have a circumglobal presence in tropical and warm temperate seas, after a taxonomic review based on integrative data (Faria et al., 2013). However, our understanding of the distribution of the Largetooth Sawfish was historically based on direct observations, fishery records, photographic evidence, and museum specimens (Fernandez-Carvalho et al., 2014). Nowadays, records of this Critically Endangered species in the wild are extremely rare, which has led to the application of non-invasive monitoring tools, such as environmental DNA (eDNA) to detect the presence of individuals in their natural habitats.

Environmental DNA (eDNA) analyses have shown potential for monitoring elasmobranch species using metabarcoding (Boussarie et al., 2018; West et al., 2020; Budd et al., 2021; de la Hoz Schilling et al., 2024), as well as using species-specific detection methods that focus on *Pristis* species (Simpfendorfer et al., 2016; Bonfil et al., 2021; Cooper et al., 2021). However, species-specific identification through eDNA detection can produce false negatives if local geographic lineages have sequences that vary at the site of the specifically designed primers/probe (Wilcox et al., 2015). The species-specific primers and probe set available for *P. pristis* detection were developed from and for the Australian population (Simpfendorfer et al., 2016), and because there are indications of geographical population structure within *P. pristis* (Faria et al., 2013; Feutry et al., 2015), it is important to consider whether the lack of detection of this species in other regions of the world (Bonfil et al., 2021; Rodrigues et al., unpublished results) could be due to mismatches between the primers/probe and the mitogenomic sequences of the local populations to which they should anneal.

Here, we aimed to verify the phylogenetic relationships and divergence times within the Pristidae family, with a particular focus on confirming the placement and relative divergence of new *P. pristis* samples, and to test whether mutations at key sites have been detrimental to species-specific detection of *P. pristis* in the Eastern Pacific and Western Atlantic using the existing probe set. To test this hypothesis, mitogenomes were assembled as a form of superbarcode (Crampton-Platt et al., 2016) to provide regional reference sequences for *P. pristis*, potentially revealing the need to develop and test alternative regional species-specific primers for *P. pristis*.

2. Methods

2.1. Sampling

A sample of muscle tissue and stomach contents of a juvenile female *P. pristis* from the Western Atlantic (specifically from 3°23'05' S 44° 48' 40' W, Bonfim do Arari, upper part of the estuary of São Marcos Bay, Maranhão, Brazil), that was caught in a fishing net and donated by fishermen to JLSN. The sample collection and transport permit ("Sistema de Autorização e Informação em Biodiversidade" - SISBIO 60306–4) and the use of national genetic resources was registered via the "Sistema Nacional de Gestão ao Patrimônio Genético e do Conhecimento Tradicional Associado" (SisGen) under license number A9851C4. A small sample of cartilage was also taken from an old dry *P. pristis* rostrum from Talara, Eastern Pacific in Peru, a historical (1960, 65 years ago) personal item belonging to the family

of MC-T, which was fished and commercialized before the species was granted legal protected status.

2.2. DNA extraction and mitogenomic sequencing

The tissue samples were processed in independent laboratories in Brazil and Peru. All procedures were performed following initial decontamination of all materials and surfaces using bleach and UV light exposure. Tissue and cartilage samples were extracted using an adapted (addition of 20 μl proteinase K for tissue lysis) CTAB protocol (Doyle and Doyle, 1987) in decontaminated laminar flow cabinets. DNA was quantified in Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., MA, USA). Libraries were prepared following standard Illumina protocols using genomic DNA concentration of 20 ng and tagmentation times varying from 20 to 25 min, followed by fragment analysis using a Tapestation 4200 (Agilent, CA, USA). The libraries were sequenced on Illumina NextSeq 2000 platforms (Illumina, San Diego, CA, USA) using NextSeq 1000/2000 P2 reagents (100 cycles) to generate 300 bp paired-end reads (insert size varying from 459 to 526 bp).

To obtain a positive mixed-species control sample (DNA of *P. pristis* along with DNA of other species), the stomach contents were separated from the ethanol preservative by three cycles of washing and centrifugation with UV sterilized ultrapure water. Four 650 μl subsample replicates and one negative control (non-template ultrapure water) were prepared and stored in separate microcentrifuge tubes (Rosa et al., 2024).

2.3. Mitogenome assembly and annotation

Quality of the generated reads was checked using FastQC v.0.12.1 (Andrews, 2010), then trimmed to remove adapters and exclude reads shorter than 70 base pairs using TrimmomaticPE v.0.39 (Bolger et al., 2014). Complete mitogenomes were assembled with NovoPlasty v.4.3.5 (Dierckxsens, et al., 2016) with a seed of *COXI* from a *P. pristis* mitogenome (NC_039438), specifically the 5500–7656 bp positions, then annotated using the MITOS2 web server v.2.1.7 (Donath et al., 2019). All tRNAs structures were identified and visualized with tRNAscan (Chan and Lowe, 2019) with default parameters. To determine accurate limits of all ribosomal units, protein-coding genes (PCGs) and secondary structure we ran an additional annotation with MITOFish v.4.01 (Zhu et al., 2023), then aligned and visually checked against an existing mitogenome of *P. pristis* from Australia (NC_039438.1 on GenBank) using Geneious Prime bioinformatic software v. 2024.0.5 (https://www.geneious.com).

Mitogenomes were drawn in a full circle using CGviewer Server (Grant et al., 2023). The GC skew was established and added to the graph using the following calculation $(G - C)/(G + C)$. The Relative Synonymous Codon Usage (RSCU) values were calculated in MEGA X (Kumar et al., 2018) and the nucleotide diversity based on 13 PCGs of *Pristis* with a 100 bp sliding window and each step of 25 bp, was estimated with DNAsp6 (Rozas et al., 2017), the results of both analyses were plotted with RStudio (R Core Team, 2023) using the ggplot2 package (Wickham, 2016).

2.4. Phylogenomic analyses

We built a dataset containing all complete mitogenomes available on GenBank for Rhinopristiformes and included complete mitogenomes for a holocephalan (*Callorhinchus milii* Bory De Saint-Vincent, 1823) and an actinopterygian (*Tor putitora* (Hamilton, 1822)) (Table 1) as the same outgroups used by Wang et al. (2023). Only the 12 H-strand PCGs and the rRNA genes were used. Although the ND6 is a coding gene, it is on the L-Strand where there is a distinct asymmetry of base composition (Miya and Nishida,

Table 1
List of mitogenomes used for phylogenetic analyses. The codes of the mitogenomes from this study are represented in bold.

Family	Specie	Length(bp)	GenBank code
Callorhinchidae	*Callorhinchus milii*	16,769	NC_014285
Cyprinidae	*Tor putitora*	16,576	NC_021755
Glaucostegidae	*Glaucostegus granulatus*	16,547	MN783017
Pristidae	*Anoxypristis cuspidata*	17,243	NC_026307
Pristidae	*Pristis clavata*	16,804	NC_022821
Pristidae	*Pristis pectinata*	16,802	NC_027182
Pristidae	*Pristis pristis*	16,912	NC_039438
Pristidae	*Pristis pristis* (Western Atlantic)	16,807	**PV053514**
Pristidae	*Pristis pristis* (Eastern Pacific)	16,914	**PV053515**
Pristidae	*Pristis zijsron*	16,804	MH005927
Rhinidae	*Rhina ancylostoma*	17,217	NC_030215
Rhinidae	*Rhynchobatus australiae*	16,804	NC_030254
Rhinidae	*Rhynchobatus djiddensis*	16,799	NC_066688
Rhinidae	*Rhynchobatus laevis*	16,560	NC_047241
Rhinobatidae	*Acroteriobatus annulatus*	16,773	NC_068897
Rhinobatidae	*Acroteriobatus blochii*	16,771	NC_068898
Rhinobatidae	*Rhinobatos hynnicephalus*	16,776	NC_022841
Rhinobatidae	*Rhinobatos schlegelii*	16,780	NC_023951
Trygonorrhinidae	*Zapteryx exasperata*	17,310	NC_024937

The captions (legends) of each figure are as follows:

2015), and it was therefore not included in the analysis. The sequences were aligned with standard MUSCLE alignment algorithm (Edgar, 2004) and visually revised on Geneious Prime bioinformatic software. To improve accuracy of phylogenetic inference, we excluded the start and stop codons of all H-strand PCGs due to their highly conserved structure, as well as the third codon position due to the high variation that can give rise to synonymous mutations that do not have functional impact (Näsvall et al., 2023). In addition, the alignment of the H-strand PCGs was split in the first and second codon position. We used Gblock v.0.91b (Castresana, 2000) to exclude ambiguous alignments for ribosomal subunits. The segments of rRNA units and H-strand PCGs were concatenated with Phylosuite v.1.2.3 (Xiang et al., 2023). After that we defined the rRNA units, first and second codons as partitions and tested whether these partitions should be analyzed using distinct evolutionary models using Partition Finder v.2.1.1 (Lanfear et al., 2016). We used ModelFinder (Kalyaanamoorthy et al., 2017) to determine the best edge-equal evolutionary model for each partition as: rRNAs (TIM2 +F+R3), first codon (GTR+F+I+G4) and second codon position (TIM3 +F+I+R2). A Maximum Likelihood tree was generated using 1.000.000 pseudoreplicates based on *Ultrafast Bootstrap* algorithm (Minh et al., 2013) in IQ-tree v.2.3.4 (Minh et al., 2020) with an *abayes* approximation test (Anisimova et al., 2011). Furthermore, a Bayesian Inference analysis based on the same dataset and partition model was conducted with two parallel runs with 1.000.000 generations, in which 25 % of the initial tree was discarded as burn-in. Both trees generated were visualized and annotated using Figtree software v.1.4.4 (Rambaut, 2018).

2.5. Divergence time analyses

The divergence time to The Most Recent Common Ancestor (TMRCA) was estimated based on a Bayesian MCC tree that was produced in BEAST 2 (Bouckaert et al., 2019) using the dataset above. The Yule speciation prior was used for the tree prior, modeled with an uncorrelated lognormal relaxed clock (Drummond et al., 2006) with GTR+G model.

At first, two fossil calibration points were used. The first one, corresponds to the appearance of Rhinopristiformes, placed in the Cretaceous as late as the Hauteruvian (97.98–130.93 mya). For this prior we use the proposed mean Aptian age of 114.26 mya following Villalobos-Segura and Underwood (2020). The Rhinopristiformes clade includes several extinct taxa in a sister-group to the extant Rhinopristiforms with †*Iansan* at the base suggesting the divergence between extinct (†"*Rhinobatos*" *grandis*, †"*R.*" *whitfieldi*, †"*R.*" *hakelensis*, †"*R.*" *tenuirostris*, †"*R.*" *latus*, †"*R.*" *intermedius*, †"*R.*" *maronita*, †*Rhombopterygia*, †*Tlalocbatus* and †*Stahlraja*) and extant Rhinopristiformes is estimated as the Cenomanian ~99 mya (89.35–113.71 mya) (Villalobos-Segura and Underwood, 2020).

In addition to these two fossil priors, we implemented the proposed prior of Wang et al. (2023) for the common ancestor of *P. clavata* and *P. pristis* ~55.3 mya (44.1–58.6 mya). The MCMC method was used to infer the divergence times with four independent runs with 120 million generations through four simultaneous runs containing four chains (one cold and three heated) with sampling performed every 1000 generations. Only runs with ESS values equal to or greater than 200 for all marginal parameters were used. The log-likelihood files generated from each run were viewed in Tracer v. 1.5 (Rambaut and Drummond, 2009), to check if the ESS values were equal to, or greater than, 200 after discarding 10 % of the trees as burn-in. The consensus MCC tree was then generated using the TreeAnnotator v. 1.4 (Drummond and Rambaut, 2007).

2.6. Species-specific eDNA probes

The previously published species-specific primers and probes targeting *P. pristis* (Cooper et al., 2021; Cooper et al., 2022) were aligned with mitogenomes available in GenBank and others generated in this study using Geneious Pro R10 (Kearse et al., 2012) with the "multiple alignment" function to identify potential mismatches susceptible to lead to false negative detection. Additionally, sequences from the target species as well as from closely related Pristidae species and potentially co-occurring (in the western Atlantic) and/or closely related elasmobranch species deposited in GenBank (Appendix A, Table A.1) were downloaded and also aligned using the "multiple alignment" function available on Geneious Pro R10 (Kearse et al., 2012). Conserved fragments within the *P. pristis* sequences, showing variation with non-target species, were identified, and a new set of species-specific primers and probe targeting a 151 bp fragment of the mitochondrial 12S region of *P. pristis* were designed using the "primers" design function of Geneious (Brys et al., 2021; Mauvisseau et al., 2021; Bommerlund et al., 2023). In addition to the visual alignment with sequences from target and non-target species used to design the assay, its specificity was further confirmed using the NCBI primer-blast function, with both "Forward and Reverse primers", "Probe and Reverse primer", and "Forward primer and reverse Probe fragment" combinations. *In-vitro* testing was conducted using DNA extracted from stomach contents of the juvenile sample from the western Atlantic as well as previously collected predominantly near-shore eDNA samples from the Brazilian Amazon Coast (BAC) (Appendix A, Table A.2) from which multiple elasmobranch species (but no Pristidae) have been identified by metabarcoding using general *COXI* primers (Rodrigues et al., unpublished results). Undiluted DNA extracts from the stomach content (5 DNA extracts analysed in duplicate) were first used to confirm that the primers/probe could amplify the target DNA. Then, a 1:1000 diluted DNA extract from stomach content was used as positive control when analyzing eDNA samples. This was done to avoid saturating the ddPCR reading with high quantification results, and to decrease potential contamination due to highly concentrated DNA template. The analysis of these two types of sample was conducted to confirm the amplification of target material in mixed-species DNA samples (DNA of *P. pristis* within its stomach contents) using the newly developed assay, and to assess as best as possible the assay's specificity. It was expected that a significant proportion of the many pre-existing near-shore eDNA sampling locations should not show presence of *P. pristis* and therefore act as negative controls by registering the absence of amplification of local non-target species diversity. This was done to mitigate challenges in obtaining genetic material needed for further laboratory validation because all Pristidae are CITES listed, which limits sharing genetic material across countries.

DNA extracted from the stomach content was analyzed using both the existing species-specific primer and probe set (Cooper et al.,

2021) as well as the newly developed primer and probe set on a Bio-Rad QX200 ddPCR System. eDNA samples were later analysed on the same ddPCR platform using only the primers/probe developed in this study. ddPCR reactions were performed in a 20 μl final volume, consisting of 10 μl Bio-Rad ddPCR supermix for probes (no dUTP), 0.75 μM of forward and reverse primers, 0.375 μM of probe, 5.0 μl of ddH2O and 2 μl template DNA. For each reaction, droplets were generated using a DG8 Droplet Generator Cartridge and 70 μl of Droplet Generation Oil for Probes on a QX200 Droplet Generator (Bio-Rad), and a final 40 μl volume of droplets for each reaction was transferred to a ddPCR 96-well plate. End-point PCR amplifications were performed on a BioRad CFX96 Real-Time System (Bio-Rad Laboratories, California, United States). PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles of denaturation for 30 s at 94°C and annealing at 55°C for 1 min, with ramp rate of 2 °C/s, followed by 10 min at 98°C and a hold at 8°C for our newly developed primers/probe set. Similar PCR conditions with an annealing temperature of 60°C were used with the primers/probe set described in Cooper et al. (2021). Droplets were read on a QX200 droplet reader (Bio-Rad), and quantification was achieved using the Bio-Rad QuantaSoft software (v.1.7.4.0917). Thresholds for positive signals were determined according to QuantaSoft software instructions, and all droplets above the fluorescence threshold were counted as positive events, those below it being counted as negative events.

3. Results

3.1. Mitogenomic variation

The sequencing depth achieved was of ~6x total genome coverage, with much deeper coverage across most of the mitogenome. The new complete mitogenome of *P. pristis* from the Western Atlantic has a total length of 16,807 bp, with the following nucleotide composition: T(U), 28.3 %; C, 26.6 %; A, 32.1 %; G, 13.0 %, resulting in an A+T content of 60 % and C+G of 40 %. In comparison, the mitogenome from *P. pristis* from the Eastern Pacific is 16,914 bp long with the following composition: T(U), 28.2 %; C, 26.7 %; A, 32.1 %; G, 13.1 %, also showing an A+T and C+G proportion of 60 % and 40 %, respectively (Fig. 1). The mitochondrial arrangement also follows the typical vertebrate pattern, containing 2 rRNA genes, 13 protein-coding genes, 22 tRNA genes (see tRNAs structures in Appendix B), and a non-coding control region (*D-loop*). However, individuals from the Eastern Pacific and Australia display a distinct feature: both have 23 tRNA genes due to the duplication of the tRNA-Pro located between the tRNA-Thr and the control region. In contrast, the mitogenome of *P. pristis* from the Western Atlantic lacks this duplication, marking a significant distinction compared to the other *P. pristis* mitogenomes described so far (Fig. 1). RSCU values showed little variation following sawfish patterns and nucleotide diversity indicates somewhat less variation in the three COX gene units compared to ATPase and NADH units at the genus level (see

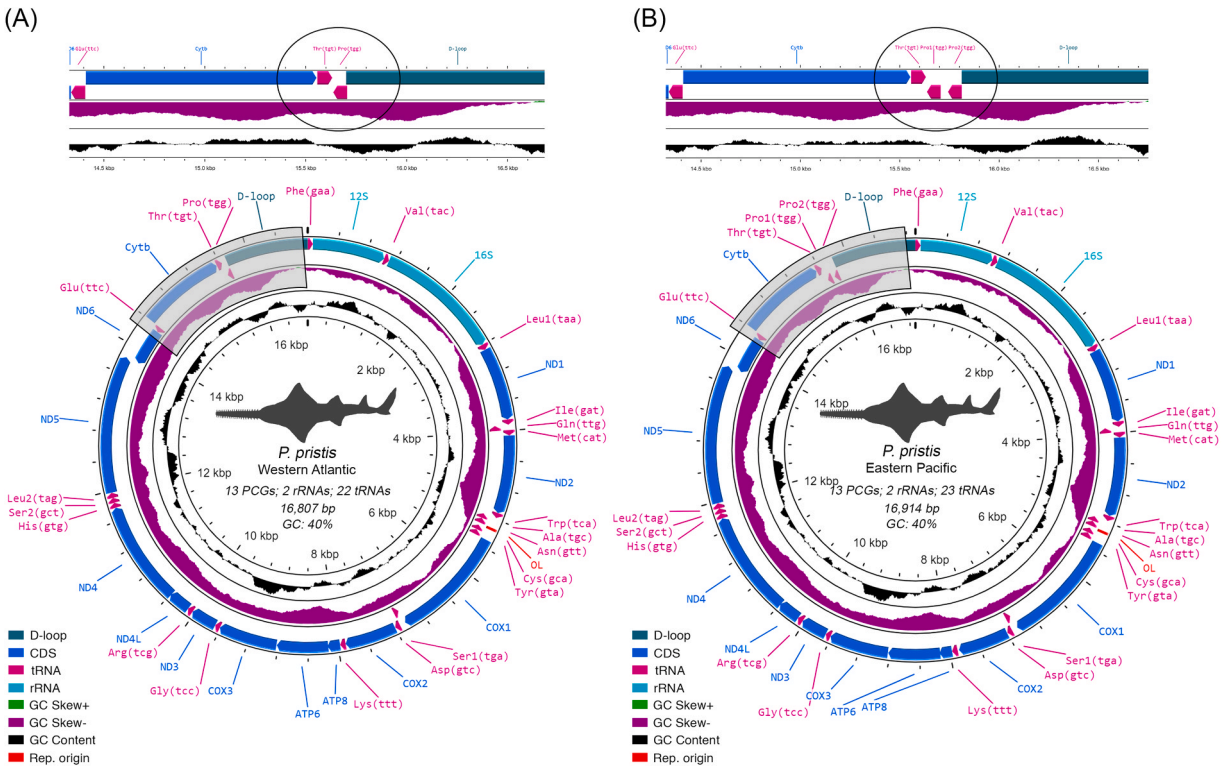

Fig. 1. Mitogenomes of new and geographically distinct samples of P. pristis with highlighted structural differences:(A) Mitogenome from the Western Atlantic sample. (B) Mitogenome from the Eastern Pacific sample.

Appendix B, Fig. B.1-B.3).

3.2. Phylogenetic relationships of Rhinopristiformes and divergence time estimations

All branches of the phylogenetic trees inferred from the 12H-strand PCGs and ribosomal units of all Rhinopristiform species were well supported (Appendix C, Fig. C.4 A, 4B), confirming the monophyly of the five families analyzed, diverging only in the position of *Glaucostegus granulatus* (Cuvier, 1829). However, the topology obtained reinforces the complete resolution of the family Pristidae. Moreover, within *P. pristis*, it confirms evidence of geographic variation between the Australian, Eastern Pacific, and Western Atlantic samples supported by both ML and Bayesian analyses (Appendix C, Fig. C.4 A, B).

The Maximum Clade Consensus tree (Fig. 2) shows a similar topology to that of Wang et al. (2023). However, we recovered differences in the estimated time since divergence between the outgroups utilized, estimated as ~150.85 mya (HPD 95 %: 155.20–182.41 mya, Fig. 2) and the most common recent ancestor of outgroups and Rhinopristiformes which was estimated at ~168.13 mya (HPD 95 %: 155.20–182.441 mya) instead of ~310.93 and ~361.83 mya, respectively, predicted by Wang et al. (2023). All fossil dates used as calibration priors are concordant with the previous literature that they were based on, especially Nodes A (Rising of Rhinopristiformes) and B (speciation between extinct and extant Rhinopristiformes). The common ancestor of Pristidae (*Anoxypristis* and *Pristis*) was estimated to exist ~71,74 mya (HPD 95 %: 66.06–77.21 mya, Fig. 2), compatible with the estimate of Wang et al. (2023) (~76.42 mya) considering the confidence intervals. A very small difference was recovered in reference to calibration Node C (common ancestor of *P. clavata* and P. *pristis*). We recovered ~54.19 mya, instead of ~55.13 from Wang et al. (2023), but with a wider interval of L HPD95 % and U HPD95 % resulting in overlap with the estimate of Wang et al. (2023).

3.3. Species-specific eDNA probes

The alignment of the previously published 12S *P. pristis* species-specific primers and probe set (Cooper et al., 2021) with the new mitogenomes identified one mismatch on the probe sequence for both new mitogenomes (Fig. 3A; Appendix D, Fig. D.2 A), one mismatch with the reverse fragment for the mitogenome obtained from the Western Atlantic specimen (Appendix D, Fig. D.2B), and two mismatches for the mitogenome obtained from the Eastern Pacific specimen (Fig. 3B; Appendix D, Fig. D.2B). We were not able to

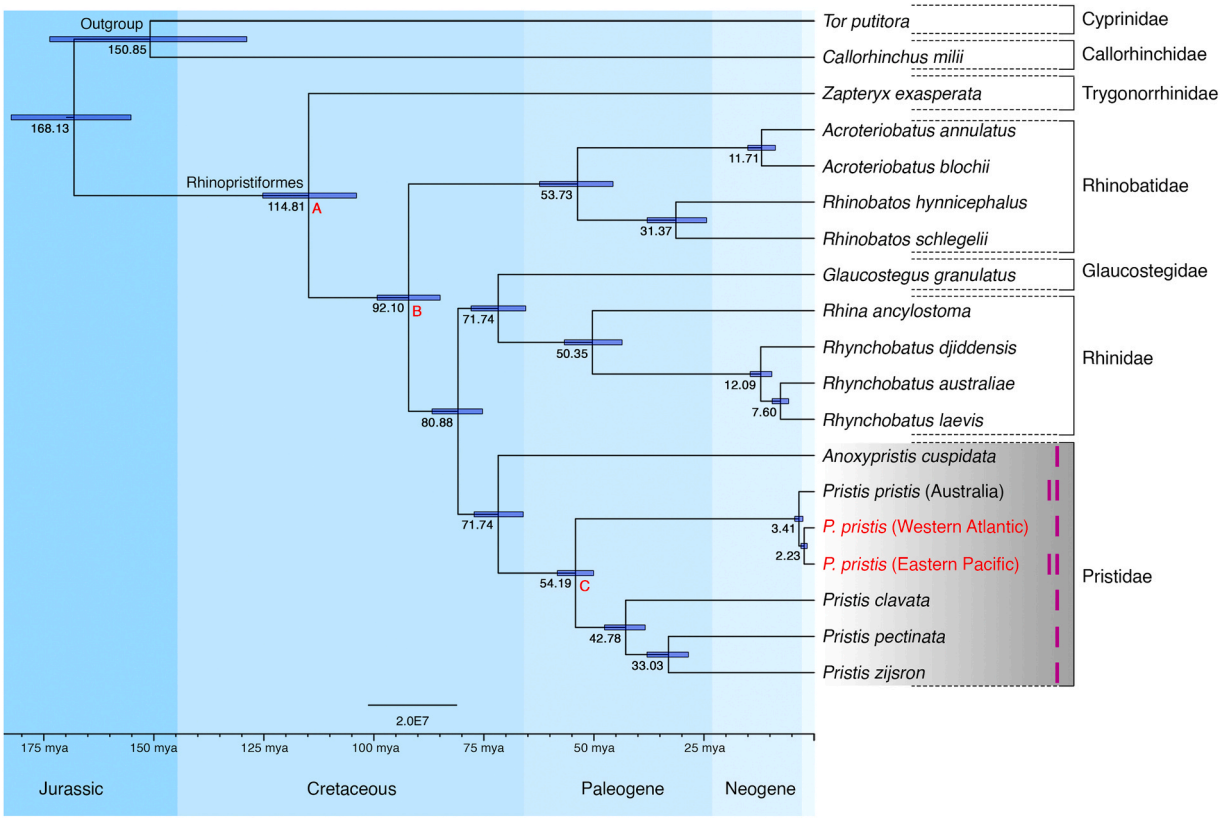

Fig. 2. Maximum clade consensus tree including divergence time estimates for Rhinopristiformes generated using the 12 H-strand PCGs and rRNA genes inferred using BEAST2. The priors indicated at the nodes in A, B, and C represent: (A) the emergence of Rhinopristiformes, (B) speciation events within Rhinopristiformes, and (C) the most recent common ancestor of P. pristis and P. pectinata. The new P. pristis from this study are in red. Purple bars after the terminal names indicate the double or single annotation of the tRNA-Pro (TGG).

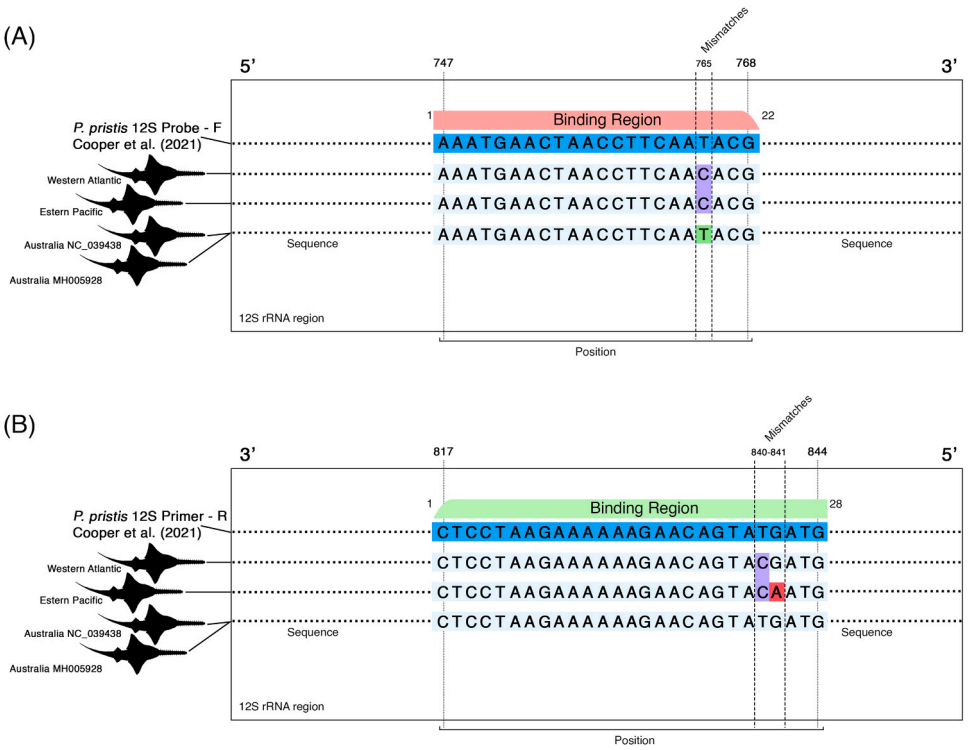

Fig. 3. Local alignment of the existing (Cooper et al., 2021) 12S species-specific Pristis pristis probe (A) and reverse primer (B) with P. pristis mitogenomes including the two new mitogenomes produced in this study for the Western Atlantic and Eastern Pacific samples as well as two Australian mitogenomes available in GenBank showing the mismatch of nucleotides for both the Western Atlantic and Eastern Pacific mitogenome lineages that prevent primer binding.

amplify the undiluted endogenous DNA extracted from the stomach content of the Brazilian specimen using these published primers and probe from Cooper et al. (2021) and obtain a positive ddPCR signal, confirming that the mismatches identified on the probe and reverse primers would lead to false negative results with *P. pristis* occurring in Brazilian waters. However, using the DNA samples extracted from the stomach content of the Brazilian specimen, we were able to amplify *P. pristis* DNA using the newly designed set of species-specific primers and probes designed in this study (Appendix D, Fig. D.6), Forward primer 5'- CCTAAGAAAAAACGAACAGTA −3', Probe 5'- CACTATTCTGAAACTGGCTC −3', Reverse primer 5'- GTTTATGTAAGGGGAATATTAT −3') (Fig. D.3-D.5; Appendix D). Due to the high concentration of *P. pristis* DNA in these samples (ranging from 6 370–12 064 copies per µl or saturating the ddPCR platform), we used a 1:1000 dilution as a positive control when analysing eDNA samples with the newly designed primers and probe. The analysis of these eDNA samples collected in the biodiversity rich marine systems of the BAC led to no detectable PCR amplification using the ddPCR platform and newly developed assay. Positive and negative controls performed as expected, therefore confirming that this assay did not amplify non-target and/or co-occurring species in this region which could lead to false positive results.

4. Discussion

4.1. Mitogenome comparisons

Both mitogenomes are highly similar to the existing mitochondrial genome of *P. pristis* described by Kyne et al. (2018) with similar GC content, nucleotide composition values and RSCU values to that and to other elasmobranch species (Kousteni et al., 2021). The slight difference in total mitogenome length between individuals from Australia/Eastern Pacific and Western Atlantic is mainly determined by the deletion of a tRNA-Pro secondary structure duplication in the mitogenome of the sample from the Western Atlantic. However, as there is currently no biological explanation for the loss of this duplication, it is necessary to investigate further, although such rearrangements on the mitochondrial genome have been reported for other bony fish species (Miya and Nishida, 1999), elasmobranchs (Winn et al., 2024; Fee et al., 2025) and other taxa (e.g., amphibians - Zhang et al., 2021, cephalopods - Yokobori et al., 2004; Taite et al., 2023).

4.2. Phylogeny and divergence time estimations

The taxonomy of the family Pristidae revised by Faria et al. (2013) based on molecular and morphological data reducing the

number of species in the genus *Pristis*, synonymizing *Pristis microdon* and *Pristis perotteti* within *P. pristis* because integrated data did not support the maintenance of these species. It was also proposed that *A. cuspidata* was basal to a *Pristis* genus clade, as follows (*A. cuspidata* (*P. pristis* (*P. clavata* (*P. pectinata*, *P. zijsron*)))) (Faria et al., 2013). Kyne et al. (2018) provided the complete mitochondrial genome of *P. pristis* and corroborated the phylogeny established for the family Pristidae (without including *P. zijsron*). Furthermore, Wang et al. (2023) added the complete mitogenome of *P. zijsron*. Our phylogenetic tree was generally consistent with the existing phylogenies using H-strand PCGs from mitogenomes (Kyne et al., 2018; Kousteni et al., 2021; Wang et al., 2023).

Pristis pristis is the sawfish with the broadest geographic distribution. Global geographical structuring of its populations in tropical Western Atlantic, Eastern Atlantic, Eastern Pacific, and Western Indo-Pacific have been detected based on divergence in NADH-2 mtDNA gene and rostral tooth counts (Faria et al., 2013). Similarly, local geographical structuring in Northern Australia has also been detected based on another region of the mtDNA, the *D-loop* (Phillips et al., 2011), as well as complete mitogenomes (Feutry et al., 2015). Given the previous geographic variation in the mtDNA and the results of this study, additional complete mitogenomes from across the full geographic range will be important to better assess *P. pristis* population structuring. However, many of the remaining specimens, particularly isolated rostra, are preserved dry in museums or private collections, which makes the sequencing particularly challenging.

The crown age for Chondrichthyes, which is indirectly estimated in our study, is concordant with previous inferences (Aschliman et al., 2012; Villalobos-Segura and Underwood, 2020). The oldest unambiguous fossil batoid remains come from open marine environments of the Toarcian (Lower Jurassic, ca. 182.7 – 174.1 mya). The variation in our estimate compared to that of Wang et al. (2023) most likely represents the effects of limited outgroup sampling (Grant, 2019) and the balance of data within the targeted ingroup considering the limitations of using only mitochondrial sequences. Both trees use the same two limited outgroups to help balance the effects of saturation (often a result of the faster rates of molecular evolution of mitochondrial DNA compared to most nuclear sequences) within the ingroup as these studies focus primarily on dating relationships within Pristidae. Reliable dating of the crown group dates for Chondrichthyes and other deeper relationships in elasmobranchs would need more genomic data and more complete taxonomic coverage of the main clades and more outgroups, and ideally include external calibration points.

The divergence estimates for *P. pristis* from the Western Atlantic, Eastern Pacific and Australian regions reveal that the variation in mitogenomic sequences represents a historical process. Global connectivity may only be possible in species with large-scale horizontal dispersal if they also tolerate a broad range of environmental conditions and/or are capable of extensive vertical movement (Hirschfeld et al., 2021). In elasmobranchs, larger species are more likely to maintain genetic connectivity across barriers related to ocean bathymetry (Stevens et al., 2014). However, there are additional factors to take into account besides body size where, habitat and maximum depth of occurrence is key factor to maintain genetic connection in transoceanic species (McFarlane and King, 1979; Weigmann, 2016). On the other hand, elasmobranchs with a larger depth distribution and that inhabit oceanic habitats are less likely to show genetic differentiation across depth and mid ocean barriers compared to species that are associated with the seafloor of continental shelves. But this trend may be reversed for shallow straits (Hirschfeld et al., 2021). In addition to these factors, there is also physiological tolerance to environmental conditions. This determines the capacity of elasmobranchs to disperse across potential barriers, especially to strong gradients of salinity which in turn, is critical to maintain connectivity across headline barriers. (Feutry et al., 2015; Bernard et al., 2016).

The separation between *P. pristis* lineages detected in the present study take place during the Glacial – Interglacial cycles of the Pleistocene (Kashiwagi et al., 2012) when considerable variation in upwelling intensity and productivity occurs during this period (Pedersen, 1983; Shaari et al., 2013). Additionally, this period was marked by a major decrease in sea level during glacial periods which may have further reinforced the isolation of populations by causing additional barriers to dispersal through the restriction of shallow seaways, especially in the Indo-West Pacific Ocean Coral Triangle (Pillans et al., 1998) which can increase the isolation between the Indo-West Pacific *P. pristis* in relation of the ancestor of the American lineages. The separation between Pacific and Western Atlantic lineages proposed in the present study must be influenced by the rising of the Panama Isthmus. The emergence of the Isthmus of Panama was a long process that caused profound but gradual changes in a range of oceanographic conditions, including temperature, salinity, circulation and productivity (O'Dea et al., 2016). The seaway is understood to have been shallowing by the Middle Miocene, decreasing in depth from over 2000 m to less than 1000 m deep (Coates, 1997). Around 4 mya, the narrowing of the seaway began to extinguish Caribbean upwelling and the primary productivity of this region dropped dramatically, while it increased in the Eastern Pacific (Coates and Stallard, 2013).

In this sense, we suggest that the vicariance between Eastern Pacific and Western Atlantic of *P. pristis* was a consequence of these physical and environmental barriers. The Isthmus of Panama Barrier was responsible for several marine species (Lessios, 2008; Lima et al., 2020, Costa et al., 2021) including elasmobranchs species with migratory capacities (Daly-Engel et al., 2012; Poortvliet et al., 2015; Sales et al., 2019). The reduced sea level together with a physical barrier can act even stronger as a vicariant force for limited coastal species like batoid (Carpenter et al., 2011; Puckridge et al., 2013).

4.3. Challenges and perspectives for species-specific eDNA monitoring of P. pristis

It is clear that the geographical genetic variation described above can severely impact the chances of generally applying species-specific detection systems developed only based on data from one region. The probes previously developed by Cooper et al. (2021) for the Australian population of *P. pristis* did not work for the species-specific detection of *P. pristis* from the Western Atlantic using our positive control. This confirms that the mismatches in the probe and reverse primer (Fig. 3) associated with these geographic mitogenome variants are most likely responsible for false negatives when attempting to detect these individuals in natural environments of the Western Atlantic using ddPCR (Bonfil et al., 2021; Rodrigues et al., unpublished results). However, it should be noted that false

negative results could also result from highly degraded eDNA template, particle adsorption, PCR inhibitors, lack of appropriate replication, or insufficient volume of water filtered (Ficetola et al., 2015; Schabacker et al., 2020; Burian et al., 2021; Mauvisseau et al., 2022; Anmarkrud et al., 2025). The new primers and probe developed here successfully amplified the target species' DNA within a mixed-species DNA sample (the stomach content samples from *P. pristis* from Brazil). Indeed, as highlighted in Thalinger et al. (2021), when following the validation scale to determine the readiness of eDNA assay for routine species monitoring, it is essential to perform *in-vitro* validation of the assay. For this, the assay should be tested against closely related and co-occurring nontarget taxa to ensure its specificity, ideally using tissue-derived DNA samples from multiple individuals spanning a defined geographic area to ensure that the assay is robust to genetic variants of local target and non-target species (Thalinger et al., 2021). In that regard, using synthetic DNA fragments using GenBank records would have prevented us from assessing the effects of genetic variants across populations. Future studies should therefore follow the testing and validation scale developed by Thalinger et al. (2021) and perform *in-vitro* testing that includes DNA from local target and non-target species. Following this, a dilution series of synthetic DNA fragments should be performed to assess the Limit of Detection and Limit of Quantification of the developed assay, in order to investigate its sensitivity (Bagdonaitė et al., 2025). As rare species are often associated with low levels of eDNA, this would allow to estimate the probability of obtaining false negative results due to assay sensitivity. Nevertheless, the lack of positive detection in eDNA samples from the region supports the specificity of the assay and indicates that the search for positive signals of these species will require greater coverage of environmental space and/or improvement of sampling methodologies that increase the chances of recovery of *Pristis* DNA (e.g., stratified sampling of the water column). In support of Lehman et al. (2020), our results further demonstrate that overcoming mitochondrial gene variations among populations of a species (Rubinoff, 2006) requires *in-silico* tests with positive and negative controls to ensure highly reliable validation of probes developed for each local population.

The primers and probe developed in this study may serve as an improvement for eDNA detection approaches and help make them work for local populations and result in more reliable tools for detection and conservation of *P. pristis*, a flagship species in the coastal areas of the Eastern Pacific and Western Atlantic. This may be especially important along the BAC, which is believed to be one of the last population refuges for *P. pristis* in the Western Atlantic (Fernandez-Carvalho et al., 2014; Nunes et al., 2016; Feitosa et al., 2017, Fordham et al., 2018; Fordham et al., 2018). This perspective of an improved ability to detect the Critically Endangered *P. pristis* in the region (present study) aligns with Bohmann et al. (2014), who emphasized the crucial importance of mapping species' occurrence, identifying important ray areas for conservation, and monitoring changes in its distribution. This approach could also support the implementation of targeted protection measures, enabling more effective conservation efforts of the remaining populations, particularly in areas under anthropogenic pressure, such as fishing and habitat degradation. Moreover, accurate species detection can contribute to global conservation initiatives, aligning with international efforts to prevent its extinction (Dulvy et al., 2014).

Populations of *P. pristis* have undergone significant decline, primarily due to overfishing (especially as bycatch), habitat loss and illegal capture to remove the rostrum/teeth (Thorson, 1982; Cavanagh et al., 2005; Palmeira et al., 2013; Dulvy et al., 2016; Moore, 2017; Cabanillas-Torpoco et al., 2023). Australia remains one of the last refuges for four of the five existing sawfish species, making it a crucial region for conservation of these species (Morgan et al., 2017; Kyne et al., 2021). In the Western Atlantic, the *P. pristis* population is believed to be restricted to coastal zones in some states of Northern and Northeastern Brazil, with occurrence of juveniles in the Amazon, Mearim River basins and Maranhão Gulf according to historical data (Faria and Charvet-Almeida, 2008; Fernandez-Carvalho et al., 2014; Nunes et a., 2016; Feitosa et al., 2017). Although fishing for this species is prohibited under Brazilian law, there are still reports of capture its by artisanal and industrial fishers (Schmid and Giarrizzo, 2017) in the region and the sale and consumption of *P. pristis* meat continues, often under names that obscure the true origin of the product (Palmeira et al., 2013; Grant et al., 2021). Occurrence data for the Eastern Pacific region is even more uncertain. Records from 2014 and 2015 indicate its continued presence in the region in Peru, highlighting the need to identify and protect critical habitats that could contribute to sawfish conservation (Mendoza et al., 2017; Cabanillas-Torpoco et al., 2020; Espinoza et al., 2022). While we used all *P. pristis* sequences available on NCBI when designing the new primers and probe to ensure a reliable amplification of the target species globally, our study demonstrates that specific subpopulations can have mismatches preventing accurate eDNA detection, and leading to false negative results using previously developed assays. It is therefore possible that unknown or unsequenced subpopulations could have mismatches preventing their amplification using our newly developed assay.

5. Conclusion

The global decline of sawfishes follows the general trend observed for many elasmobranchs, with the aggravating factor that they are more likely to die as bycatch in artisanal coastal fisheries, since their rostra easily become entangled in nets and are also considered high-value items in the illegal market. We have shown that species-specific monitoring methods using eDNA, which has been developed based on genetic knowledge from a limited geographic region, may have limited generalized use in other regions. The new mitogenomes and species-specific ddPCR primers and probe set developed here may contribute substantially to improving the potential to map and monitor these Critically Endangered species across the globe.

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Ethical statement

All procedures involving animals were conducted in accordance with relevant institutional and national guidelines. Prior approval was obtained through the "Sistema de Autorização e Informação em Biodiversidade" (SISBIO) under license number 60306–4, and the "Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado" (SisGen) under license number A9851C4. We followed the appropriate guidelines for reporting animal research, and no endangered species were harmed. The samples used in this study were obtained without violating any current law or guideline.

CRediT authorship contribution statement

AESR and RMSB should be considered co-1st authors. Alan Érik S. Rodrigues: Data curation, Formal analysis, Investigation, Writing – original draft (lead), Writing – review & editing (equal). Rafaela Maria S. Brito: Formal analysis, Investigation, Writing – original draft, Writing – review & editing (equal). Patricia Charvet: Investigation, Resources, Writing – review & editing (equal). Vicente V. Faria: Investigation, Resources; Writing – review & editing (equal). Mariano Cabanillas-Torpoco: Investigation, Writing – review & editing (equal). Alexandre P. Aleixo: Funding acquisition, Resources, Writing – review & editing (equal). Tibério César T. Burlamaqui: Investigation, Writing – original draft, Writing – review & editing (equal). Luis Fernando da S. Rodrigues-Filho: Writing – original draft, Writing – review & editing (equal). Angelico Asenjo: Investigation, Writing – review & editing (equal). Raquel Siccha-Ramirez: Investigation, Resources, Writing – review & editing (equal). Jorge Luiz S. Nunes: Investigation, Supervision, Writing – review & editing (equal). Hugo J. de Boer: Funding acquisition, Resources, Writing – review & editing (equal). José Cerca: Writing – original draft (supporting), Writing – review & editing (equal). Quentin Mauvisseau: Conceptualization, Formal analysis, Methodology, Project administration, Resources, Supervision, Writing – original draft (supporting), Writing – review & editing (equal). Jonathan S. Ready: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft (supporting), Writing – review & editing (equal). João Bráullio L. Sales: Conceptualization, Investigation, Project administration, Resources, Supervision, Writing – original draft (supporting), Writing – review & editing (equal).

Declaration of generative AI and AI-assisted technologies in the writing process

No generative AI or AI-assisted technology was used at any stage of this work.

Declaration of Competing Interest

The authors declare that no competing interests exist.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2025.e03757.

Data availability

All the Sequence Read Archive (SRA) required to replicate this study has been deposited in a BioProject on NLM-NCBI (PRJNA1217631).

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