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Using environmental DNA to identify priority areas for sawfish conservation in Costa Rica, Central America

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ABSTRACT: Environmental DNA (eDNA) analysis is becoming a valuable conservation tool for detecting rare and threatened species in a variety of aquatic environments, but studies in freshwater and estuarine habitats with high levels of suspended sediment are still limited. This study used a large-scale eDNA survey to test for the presence of the Critically Endangered largetooth and smalltooth sawfishes *Pristis pristis* and *P. pectinata* in Costa Rica. Specifically, we used species-specific TaqMan quantitative PCR assays to test for the presence of both sawfish species in freshwater, estuarine, and marine habitats through a nationwide survey using standardized field and laboratory protocols developed by the Global Sawfish Search Project (James Cook University, Australia). A total of 558 samples were collected from 18 sites, 93 stations, and 6 geographic regions; 486 samples were used to test for *P. pristis*, 55 samples were used to test for *P. pectinata*, and 93 were included as negative controls. *P. pristis* was detected in 16 samples collected from the northern and north Caribbean regions of Costa Rica; however, there were no detections of *P. pectinata*. Our eDNA survey provides the first indirect evidence for the presence of *P. pristis* in fast-flowing freshwater habitats with high levels of suspended sediment in Costa Rica. Based on our findings, the San Juan-Colorado River basin, a natural border between Costa Rica and Nicaragua, is a key region in Central America to guide future conservation efforts for the recovery of *P. pristis*.

KEY WORDS: *Pristis pristis* · Central America · eDNA · Critical habitat · Population recovery

1. INTRODUCTION

Understanding the distribution of threatened aquatic species presents serious challenges when dealing with cryptic and bottom-dwelling species that occur at low population densities (Bellemain et al. 2016, Simpfendorfer et al. 2016). Moreover, traditional survey methods such as underwater visual census and fishing techniques may also fail to detect rare species that live in estuarine and freshwater environments with elevated suspended sediment loads (Simpfendorfer et al. 2016, Wilcox et al. 2016). Environmental DNA (eDNA) analysis has become a powerful ecological approach to detect traces of a species' DNA in the environment using quantitative PCR (qPCR) and/or high-throughput sequencing, enabling the detection of rare and threatened aquatic organisms in a wide range of habitats (Dejean et al. 2011, Thomsen et al. 2012, Laramie et al. 2015, Sasso et al. 2017). Given the potential rapid degradation of eDNA in aquatic environments, positive detection provides a strong indication of the presence of the species in a particular environment (Barnes et al. 2014). Therefore, eDNA analysis is becoming useful for identifying the critical habitats of threatened species in poorly studied aquatic environments such as large

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tropical rivers and estuarine habitats with high suspended sediment (Syvitski et al. 2014, Bellemain et al. 2016, Huerlimann et al. 2020).

The largetooth and smalltooth sawfishes *Pristis pristis* and *P. pectinata* are bottom-dwelling rays with a highly modified rostrum (14–23 rostral teeth per side in *P. pristis* and 20–32 rostral teeth per side in *P. pectinata*) that can grow to a total length of 6.5 m (Faria et al. 2013, Last et al. 2016, Carlson et al. 2022, Espinoza et al. 2022). Both species are known to occur in tropical and subtropical freshwater, estuarine, and marine habitats, and they are often referred to as euryhaline generalists (Grant et al. 2019, Carlson et al. 2022, Espinoza et al. 2022). In some countries, such as Australia, *P. pristis* spends its first 5 yr in freshwater and later moves to estuarine and coastal waters, whereas in Lake Nicaragua and the San Juan-Colorado River (Central America), the species appears to complete its entire life cycle in freshwater (Thorson 1982a, Whitty et al. 2017, Lear et al. 2019). Juvenile *P. pectinata* are restricted to freshwater and estuarine habitats, while adults occur both in estuarine and coastal waters (Carlson et al. 2022). Globally, *P. pristis* and *P. pectinata* have undergone >80% population reduction over the last 3 generation lengths (68 yr in *P. pristis* and 59 yr in *P. pectinata*) due to overexploitation, habitat degradation, and reductions in extent of occurrence, resulting in both species being listed as Critically Endangered by the IUCN Red List (Dulvy et al. 2016, Yan et al. 2021, Carlson et al. 2022, Espinoza et al. 2022). Moreover, their euryhaline generalist nature exposes them to numerous threats in both freshwater and marine habitats, contributing to their high risk of extinction (Grant et al. 2019, Yan et al. 2021).

P. pristis comprises 4 subpopulations worldwide: the Eastern Atlantic, Western Atlantic, Eastern Pacific, and Indo-West Pacific (Faria et al. 2013). *P. pectinata*, conversely, has only 2 subpopulations: one in the Western Atlantic and the other in the Eastern Atlantic (Dulvy et al. 2016, Carlson et al. 2022). These 2 species were historically found in the Central American region (Thorson et al. 1966, Thorson 1982a, Espinoza et al. 2022); however, their presence is currently uncertain in some areas due to the lack of recent studies and severe depletion by overfishing (McDavitt 2002, Dulvy et al. 2016, Yan et al. 2021). A recent study in Costa Rica suggests that the Humedal Nacional Térraba-Sierpe (HNTS) (southern Pacific region) and the San Juan River (northern region) may be 2 important hotspots for *P. pristis* in Central America (Valerio-Vargas & Espinoza 2019). Based on local ecological knowledge and landing records, Valerio-Vargas & Espinoza (2019) showed that there were 24 confirmed records of *P. pristis* in these 2 areas of Costa Rica from 2013 to 2018, while there were only 2 records of *P. pectinata* in the early 2000s. However, a detailed understanding of the specific habitats where both species occur is still limited due to their low natural abundance and apparent confinement to highly turbid freshwater and estuarine environments (Wiley & Simpfendorfer 2010, Simpfendorfer et al. 2016, Valerio-Vargas & Espinoza 2019). Moreover, there are remote areas of Costa Rica that could serve as potential sawfish habitats but which have been poorly studied, highlighting the need to identify key areas of the country where the species persists. Improving our knowledge of these areas will therefore facilitate the implementation of management and conservation measures where they are most needed (Harrison & Dulvy 2014, Fordham et al. 2018).

This study used a large-scale eDNA survey to test for the presence of *P. pristis* and *P. pectinata* in Costa Rica. To achieve this goal, we used a stratified sampling approach across freshwater, estuarine, and coastal/marine habitats to survey (1) areas with historical and current sawfish records (Thorson 1982a, Valerio-Vargas & Espinoza 2019, Espinoza et al. 2022) and (2) areas with suitable sawfish habitats where no sightings or landing records were ever recorded. Although most of our sampling efforts were focused on *P. pristis*, we also tested for the presence of *P. pectinata* in water samples collected from coastal Caribbean habitats in Costa Rica, given the historical occurrence of the species in this region and its potential to re-establish in the region following signs of population recovery in the southeastern USA (Brame et al. 2019). The use of eDNA analysis can help identify specific areas in Costa Rica where sawfish still occur, and ultimately prioritize future management and conservation efforts.

2. MATERIALS AND METHODS

2.1. Study region

Costa Rica is a small country (approx. 51 100 km^2 of land) in Central America, located between Nicaragua in the north and Panamá in the south (Cortés & Wehrtmann 2009). Costa Rica's Pacific coastline is long (1254 km) and highly irregular. Most of the mangrove cover (99%) is found in the central and south Pacific regions, mainly due to the discharge of large rivers and an intense rainy season (Jiménez & Soto 1985, Jiménez 1999). The Caribbean coastline is rel-

atively short (212 km) and less irregular, with small mangrove patches located mainly in the south (Gandoca-Manzanillo); there are some coastal lagoons associated with large rivers such as the San Juan-Colorado, Pacuare, Matina, and Sixaola (Cortés & Wehrtmann 2009). The northern region of Costa Rica is characterized by a vast network of freshwater environments including the Sarapiquí, San Carlos, and Frío rivers (Rojas 2011), which form a continuum with Lake Nicaragua and its outflow, the binational San Juan-Colorado River. This large river diverges and empties into the Caribbean Sea at 2 locations: San Juan del Norte in Nicaragua, and Barra del Colorado in the Northern Caribbean region of Costa Rica (Thorson 1982a).

2.2. Site selection

Four criteria were used to select sampling sites for eDNA surveys: (1) sites that were located in or near the 2 potential sawfish hotspots identified by Valerio-Vargas & Espinoza (2019); (2) sites that were historically important for sawfish based on previous studies and local ecological knowledge (Thorson 1982a, Angulo et al. 2013, Valerio-Vargas & Espinoza 2019); (3) sites with no sawfish records but with suitable habitats for the species, such as mud-sand flats, mangrove-lined estuaries, shallow (<10 m) bays and rivers, some of these within protected areas (see Table 1) (Simpfendorfer et al. 2011, Yan et al. 2021, Espinoza et al. 2022); and (4) sites with recent (2020–2021) sawfish reports of captures and/or landings (M. Espinoza pers. comm.). Based on these criteria, a total of 18 sites across 6 geographic regions of Costa Rica (northern Pacific, central Pacific, southern Pacific, northern region, northern Caribbean, and southern Caribbean) were chosen to conduct eDNA surveys (Fig. 1).

Sampling effort varied between sites and regions to cover as much of the country as possible, while prioritizing key areas for sawfish, such as the HNTS in the southern Pacific and the San Juan-Colorado River basin in the northern Caribbean coast of Costa Rica, which were previously identified by Valerio-Vargas & Espinoza (2019) (see Table 1, Figs. S1–S6, Table S1 in Supplement 1 at www.int-res.com/articles/suppl/n057p161_supp1.pdf). In addition, special attention was given to remote, inaccessible areas of the Pacific coast of Costa Rica with suitable sawfish habitat that had been poorly explored in the past, such as Bahía Potrero Grande in the northern Pacific, and Laguna Pejeperro and Laguna Pejeperrito in the southern Pacific (see Table 1, Figs. S1–S3 & S6). Ultimately, sampling effort depended on the accessibility and conditions of each site as well as budget constraints.

Water samples collected at all sites were used to test for the presence of *Pristis pristis*, as this species is known to occur along the Pacific, Caribbean, and the San Juan-Colorado River basin of Costa Rica (Thorson 1982a, Valerio-Vargas & Espinoza 2019). Recent records of *P. pectinata* in the Caribbean coast of Costa Rica are scarce (Valerio-Vargas & Espinoza 2019) and, based on historical observations, this species was rare in the San Juan-Colorado River basin in the 1960s and 1970s (Thorson 1982a). Therefore, only water samples collected from the lower reaches of the Colorado River at Barra del Colorado (northern Caribbean) and Río Sixaola (southern Caribbean) were tested for *P. pectinata* eDNA (Fig. 1). The Laguna Gandoca in the southern Caribbean was not tested for *P. pectinata* because it is only temporarily connected to the coast and was only recently opened at the time of sampling (Fig. 1). Site-specific information on protection status, habitat type, and season was also identified for all sites (Table S1)

Using satellite images, we first created a set of sampling stations within each main site using a stratified sampling design in which the stations were fixed and equidistantly spaced within sites. The number of sampling stations and distance from each other was set depending on the size and relative importance of each of the main sites, as we aimed to maximize sampling area coverage across regions. Therefore, the distance between stations varied from 150 m to 8.5 km in some sites. Once in the field, the location of each station was further refined based on traditional ecological knowledge, potential suitable sawfish habitat, recent catch or sighting information, or accessibility (Figs. S1–S6). However, efforts were made to maintain an equidistant sampling strategy between subsites. All main sites were sampled from February to August 2019, during dry (November to April) and rainy (May to October) seasons. Some of the stations in the San Juan River were sampled in March (dry season) and others in May (rainy season).

2.3. eDNA sampling

Samples for eDNA analysis were collected from an anchored boat using protocols developed by Cooper et al. (2021). Before the sampling procedure, we recorded the latitude and longitude (Garmin GPSMAP 62s), water depth (Hondex depth meter),

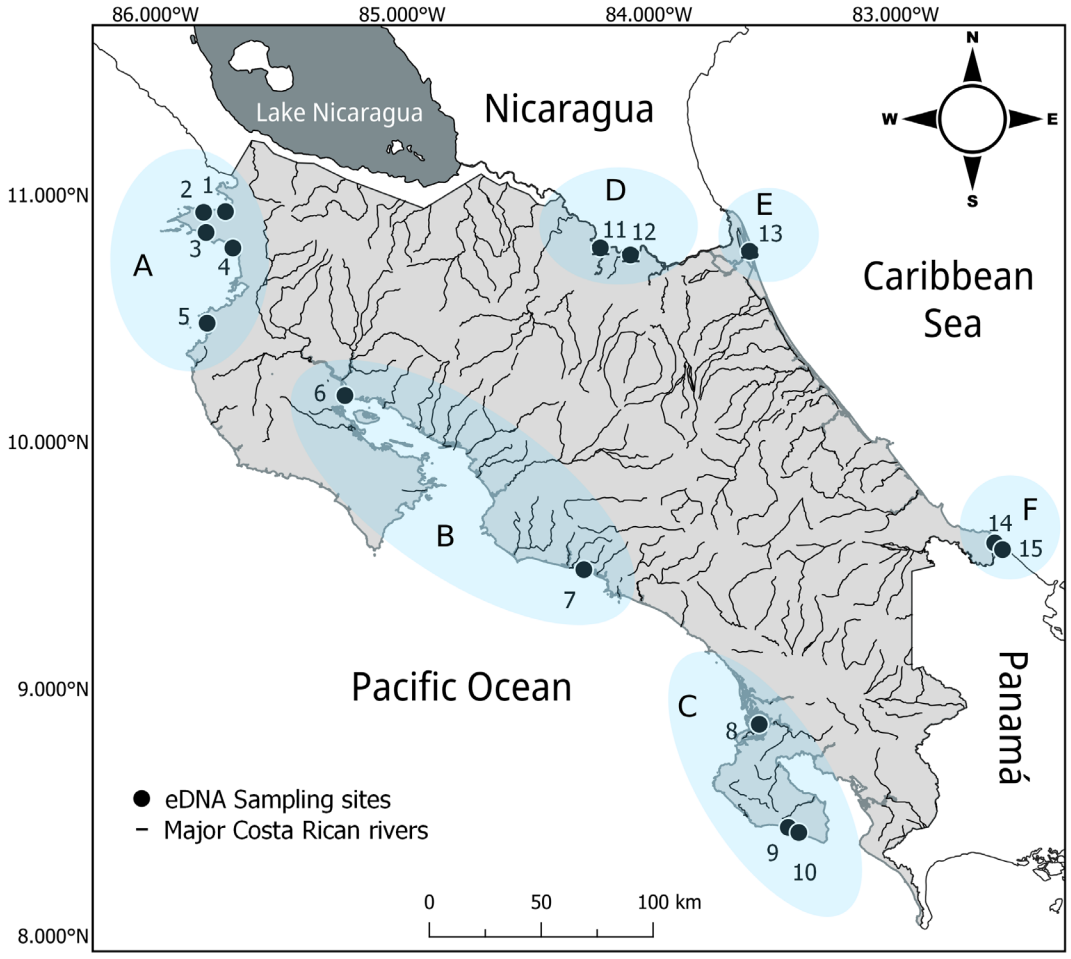

Fig. 1. Location of environmental DNA (eDNA) sampling regions (light blue shading) and sites (black circles) used to survey the presence of the largetooth sawfish *Pristis pristis* in Costa Rica, Central America. Regions include (A) northern Pacific, (B) central Pacific, (C) southern Pacific, (D) northern region, (E) northern Caribbean, and (F) southern Caribbean. Sites within regions include (1) Bahía Thomas, (2) Bahía Santa Elena, (3) Bahía Potrero Grande, (4) Bahía Naranjo, (5) Playa Danta y Dantita, (6) Boca Tempisque, (7) Estero Palo Seco, (8) Humedal Nacional Térraba-Sierpe, (9) Laguna Pejeperrito, (10) Laguna Pejeperro, (11) Boca San Carlos, (12) Boca Cureña-Cureñita, (13) Río Colorado (Barra del Colorado), (14) Laguna Agua Dulce (Barra del Colorado), (15) Black Lagoon (Barra del Colorado), (16) Dos Bocas (Río Colorado), (17) Laguna Gandoca, and (18) Río Sixaola. Sites 13 and 15 were tested for *P. pectinata* eDNA

turbidity (Secchi disc), and flow speed (water flow at each station was visually categorized as slow, moderate, or fast) of the body of water. A handheld multimeter (EcoSense EC300A) was used to record water temperature (°C) and salinity (ppt). Nearly 10% of water temperature measurements were collected with a data logger (HOBO UA-002-08) due to multimeter failure; no data on salinity was recorded in those same instances. Prior to sample collection, the working area in the boat and sampling equipment, including all environmental equipment that went into the water, were sterilized by applying a 10% bleach solution using gloves and a saturated paper towel, leaving the bleach to stand for 5 min, followed by cleaning with distilled water using a moistened paper towel (Cooper et al. 2021).

At each station, the aim was to filter a total of 5 replicate 5 l water samples (25 l in total for each station), plus a negative control using 1 l store-bought bottled water. The negative control was treated in the same way as the field samples collected. As all sampling stations were relatively shallow (<10 m deep), water samples were taken directly at the surface using a micro electric diaphragm water filter pump (12VDC 80W GOOD PUMPs) and a plastic hose submerged 50 cm. Nylon membranes with pore sizes varying from 5 to 20 µm were used to filter water samples collected at each site (see Table 1). This pore size was chosen to allow the proposed amount of filtrate in each scenario but was dependent on the turbidity of the water; turbid water was filtered with a larger pore size than clear water (Turner et al. 2014, Simpfendorfer et al.

2016, Cooper et al. 2021). Although the ideal volume of water filtered at each station for eDNA analysis was 25 l, this was not always possible due to excess sediments in some stations. Information on the filtrate amount, membrane pore size, and total filtering time was recorded for each replicate (see Table 1, Dataset S1 in Supplement 2 at www.int-res.com/articles/suppl/n057p161_supp2.xlsx). A negative field control was included at every station, consisting of the filtration of 1 l of store-bought bottled water to test for potential cross-contamination. Following filtration, each filter was rolled and folded in half using sterile forceps and scissors and placed inside a 2 ml LoBind microtube containing 1.5 ml of Longmire's buffer solution. Samples were stored at ambient temperature during the survey period and shipped to James Cook University, Queensland, Australia, where they were stored at ambient temperature until extraction in a dedicated environmental DNA extraction laboratory.

2.4. Laboratory analysis

Extraction and purification of total eDNA was completed following a glycogen-aided precipitation extraction method, described in Edmunds & Burrows (2020) with additional modifications for filter papers stored in 2 ml microtubes (Cooper et al. 2021). An extraction blank was included with every extraction batch (i.e. an extraction blank per 29 samples, per centrifuge rotor capacity). For eDNA extraction, Longmire's buffer from each 2 ml field tube was transferred to a DNA-free 15 ml LoBind® conical tube (Eppendorf), diluted to 5 ml with UltraPure distilled water (ThermoFisher Scientific), and precipitated overnight at 4°C with 7 ml isopropanol, 10 µl glycogen (20 mg ml^{-1}; Merck), and 1.7 ml of 5 M sodium chloride. The precipitate was pelleted and resuspended in 600 µl lysis buffer (0.8 M guanidine hydrochloride, 0.5% TritonX pH 10) along with the original filter paper half in the field tube, then subjected to freeze–thaw lysis and incubated at 50°C for ≥3 h (Lever et al. 2015). eDNA was precipitated overnight at 4°C using 2 volumes of polyethylene glycol solution (1.6 M NaCl, 30% polyethylene glycol) and 5 µl glycogen, then pelleted at 14 000 × *g* for 30 min (Centrifuge 5430R, Eppendorf). Final pellets were washed twice with 70–75% ethanol and eluted in 100 µl UltraPure water in 2 ml LoBind® microtubes (Eppendorf) (Lecerf & Le Goff 2010).

Presence-only detection of *P. pristis* (95% limit of detection [LOD]: 1.25 copies per reaction) and *P. pectinata* (95% LOD: 5 copies per reaction) was conducted using species-specific TaqMan qPCR assays on Applied Biosystems QuantStudio 5 Real-Time PCR System (Life Technologies, ThermoFisher Scientific) (Cooper et al. 2021). A TaqMan Exogenous Internal Positive Control (IPC) qPCR assay (Applied Biosystems; Hartman et al. 2005) with a custom internal probe modification (i.e. ABY-QSY) was used to test for qPCR inhibition. The IPC assay was included in optimized multiplexed reactions for 3 eDNA samples from every station. Samples with partial or complete inhibition were identified by examining cycle threshold (C_t) values and applying the IPC threshold of $\Delta C_t \geq 3$ to amplification curves using QuantStudio 5 qPCR System Software. Inhibited samples were diluted 1:2 and 1:10 sequentially, if indicated, using UltraPure distilled water, and qPCR analysis was repeated until inhibition was resolved.

Reactions were run in 384-well plates and contained triplicate no-template controls (NTCs), associated extraction blanks, and low-copy synthetic DNA standards (10, 5, 2.5, and 1.25 copies per reaction) as positive controls. Species-specific synthetic DNA fragments (sDNA; gBlocks™; Integrated DNA Technologies) were based on a consensus of 12S nucleotide sequences for each species and contained a mid-sequence modification (8–12 bp reversal) to permit differentiation of sDNA and eDNA amplicons via Sanger sequencing as a cross-contamination control. Putative-positive amplicons were visualized on 1.5% agarose gel and compared with a DNA electrophoresis ladder to confirm correct amplicon size. All amplicons were matched to the target sequence and sent to the Australian Genome Research Facility for clean-up and bidirectional Sanger sequencing. Sequences were aligned and trimmed, and the final consensus sequence was compared to all other compiled 12S sequences in Geneious (v.10.2.6).

All laboratory procedures were completed in dedicated, physically separated rooms for low-copy eDNA extraction, pre-PCR, and post-PCR processes. The sequential, unidirectional workflow minimises the risk of cross-contamination. Extraction, qPCR preparation, and amplification of eDNA took place in the eDNA extraction, pre-PCR, and post-PCR rooms, respectively. Plates were sealed prior to leaving the pre-PCR room and loading into the QuantStudio qPCR system in the post-PCR room.

2.5. Data analysis

QuantStudio Analysis Software (v.1.4.2) was used to analyse the C_t value based on automatic base-

line and manually determined threshold fluorescence values (0.7 ΔRn). Samples were then assigned a binomial indicator (1 or 0) to indicate presence or absence, respectively, for qualitative interpretation of the presence of target sawfish species eDNA in each sample. Sequence data were aligned in Geneious (v.10.2.6), and species identity was confirmed using the BLAST function against the GenBank NCBI database. Inter-species sequence variation was analyzed by visual assessment of aligned sequence data in Geneious (Kearse et al. 2012). Detections were considered true positives if the following selection criteria were met: (1) ≥1 technical replicate exhibited amplification that crossed the fluorescence threshold within 50 cycles; (2) amplicon produced by subsequent end-point PCR exhibited a single band of expected size; (3) BLASTn search of sequences matched target species with ≥97% pairwise identity; and (4) corresponding field and extraction blanks, and NTCs, exhibited no amplification (Cooper et al. 2021). To examine the spatial distribution of sawfish across sampling sites and stations, we plotted all confirmed positive records using QGIS (v.3.18) (https://www.QGIS.org).

3. RESULTS

3.1. eDNA detection

A total of 558 samples were collected from 18 sites, 93 stations, and 6 geographic regions of Costa Rica (Fig. 1, Table 1). Of these, 465 samples were used to test for *Pristis pristis* and 55 samples were used to test for *P. pectinata*; 93 samples were used as negative controls. The volume of water filtered across stations varied from 5 to 25 l (mean ± SD: 18.6 ± 6.6 l). A total of 353.8 l of water was filtered using 5 µm pore size filters (16.1 ± 10.2 l across stations); 550.3 l of water

Table 1. Summary of environmental DNA (eDNA) samples collected from 18 sites in 6 geographic regions of Costa Rica, Central America. Volume 5 µm, 10 µm, and 20 µm: total volume filtered (l) with 5, 10, and 20 µm pore size filters, respectively; Total Vol.: total volume filtered (l) at each site. Samples with positive eDNA detections for *Pristis pristis* and *P. pectinata* are shown in the last 2 columns. In the 'No. of samples' column, (B) indicates samples with qPCR for both *P. pristis* and *P. pectinata*; NF: not found in this area

Region/site	Date (mm-yy)	Season	No. of stations	No. of samples	Volume 5 µm	Volume 10 µm	Volume 20 µm	Total Vol.	Samples with *P. pristis*	Samples with *P. pectinata*
Northern Pacific										
Bahía Santa Elena	Jul-19	Rainy	7	35	53.9	85	17.4	156.3	0	NF
Bahía Thomas	Jul-19	Rainy	2	10		43.6		43.6	0	NF
Estero Potrero Grande	Jul-19	Rainy	12	60	55	159.5	69	283.5	0	NF
Bahía Nancite	Jul-19	Rainy	3	15	75			75	0	NF
Playa Danta / Dantita	Jul-19	Rainy	5	25	125			125	0	NF
Central Pacific										
Tempisque	Feb-19	Dry	4	20			62.5	62.5	0	NF
Palo Seco	Apr-19	Dry	7	35		2	143	145	0	NF
Southern Pacific										
Humedal Nacional Térraba-Sierpe	Jan-19	Dry	11	55		67.1	155.5	222.5	0	NF
Laguna Pejeperrito	Jun-19	Rainy	6	30			131.5	131.5	0	NF
Laguna Pejeperro	Jun-19	Rainy	7	35	14.5	109.5	21.7	145.7	0	NF
Northern region										
Boca San Carlos (Río San Juan)	Mar-19	Dry	3	15			47.2	47.2	7	NF
	May-19	Rainy	1	5			15	15	2	NF
Boca Cureñita (Río San Juan)	May-19	Rainy	7	35			71.4	71.4	5	NF
Northern Caribbean										
Barra del Colorado (estuary)	May-19	Rainy	4	20 (B)		2.1	35.4	37.5	2	0
Barra del Colorado (Laguna Agua Dulce)	Jun-19	Rainy	1	5 (B)	6.4	5		11.4	0	0
Barra del Colorado (Laguna de Atrás)	Jun-19	Rainy	3	15 (B)	24	1.5		25.5	0	0
Río Colorado (Dos Bocas)	Jun-19	Rainy	4	20			34.6	34.6	0	NF
Southern Caribbean										
Laguna de Gandoca	Aug-19	Rainy	3	15		75		75	0	NF
Río Sixaola	Aug-19	Rainy	3	15 (B)			26.5	26.5	0	0

was filtered using 10 µm pore size filters (15.7 ± 9.3 l across stations); and 830.7 l of water was filtered using 20 µm pore size filters (15.1 ± 6.5 l across stations) (Table 1, Dataset S1).

Environmental DNA from *P. pristis* was successfully extracted, amplified, and sequenced from samples collected in 2 of the 6 regions sampled, including the northern region and northern Caribbean (Table 1, Fig. 2). Overall, *P. pristis* was detected in 3 of the 18 sites and 10 of the 93 sampling stations surveyed (11%). All positive samples (16 out of the 465 samples) used to test for *P. pristis* were collected with 20 µm filter membranes (Table 1). A total of 40 samples (109 l filtered) from 3 sites in Barra del Colorado and 15 samples (26.5 l filtered) from Río Sixaola were used to test for *P. pectinata* in the northern and southern Caribbean, respectively (Table 1). However, there were no detections of *P. pectinata* at any of these sites.

P. pristis was only detected in sampling stations from the northern region and the northern Caribbean coast of Costa Rica (Figs. 1 & 2). Detections in the northern region occurred along the main channel of the Río San Juan (Fig. 2A), while in the northern Caribbean, detections occurred both within the estuary and in freshwater habitats (Fig. 2B). In the northern region, 3 stations of Boca San Carlos had 9 confirmed positive detections of *P. pristis*. Of these, positive detections were obtained during the dry (7 samples; 22.2 l filtered) and rainy (2 samples; 6 l filtered) seasons. There were 5 confirmed detections of *P. pristis* at 5 stations from Boca Cureña-Cureñita (Fig. 2A), of which positive detections were obtained from 1 dry season sample (3.7 l filtered) and 4 rainy season samples (7.5 l filtered). In Barra del Colorado (northern Caribbean), there were only 2 sampling stations where 2 samples (4.3 l filtered) had confirmed detections of *P. pristis*, both during the rainy season (Fig. 2B). All of these sites, including the Barra del Colorado estuary near the mouth of the river, were characterized by freshwater conditions with high turbidity and current, and water temperatures ranging from 27.6 to 29.9°C (Table 2). Moreover, all the stations with positive detections were relatively shallow (<4 m deep), with the exception of a 9 m deep pool at Boca Cureña-Cureñita in the Río San Juan, northern region (Table 2).

Field controls, extraction negative controls, and qPCR NTCs showed no amplification for *P. pristis* and *P. pectinata* or other related species.

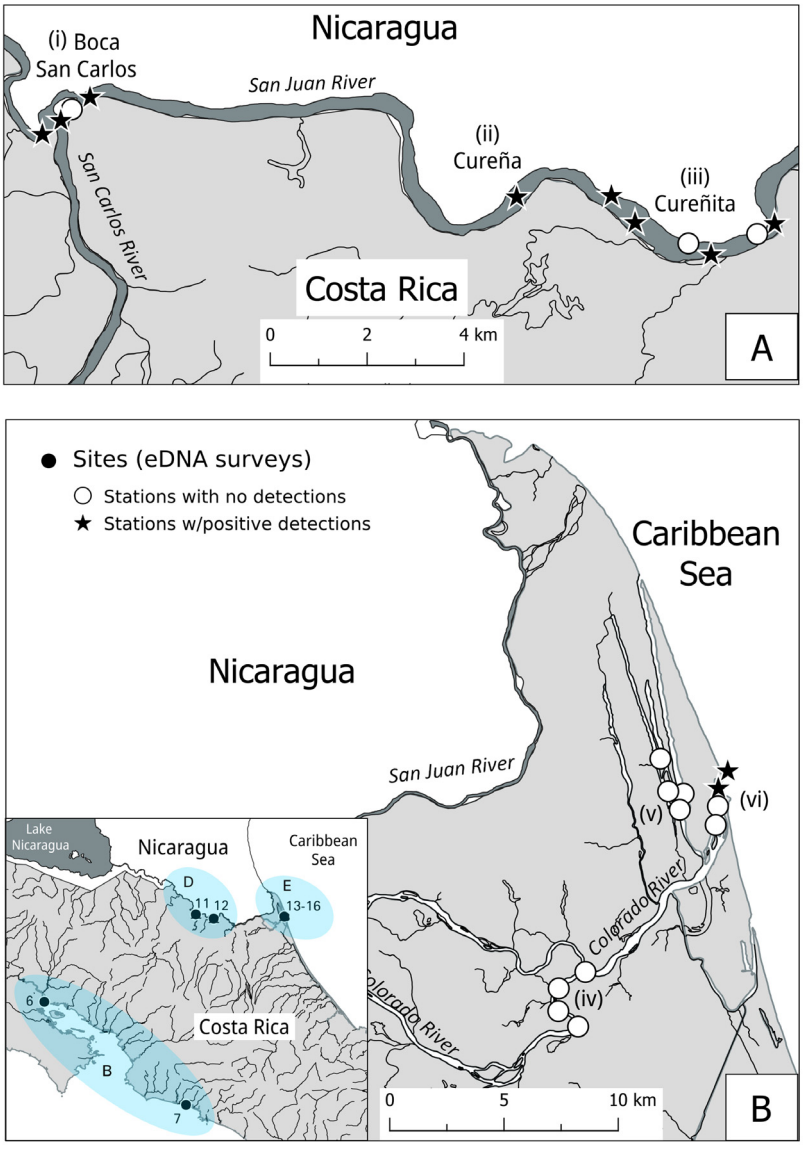

Fig. 2. Location of eDNA sampling sites and stations with positive detections for the largetooth sawfish *Pristis pristis* in Costa Rica. (A) Sampling stations from the northern region, including (i) Boca San Carlos, (ii) Boca Cureña, and (iii) Cureñita. (B) Sampling stations from Barra del Colorado in the northern Caribbean, including (iv) Dos Bocas (Río Colorado), (v) Laguna Agua Dulce and Laguna de Atrás, and (vi) the estuary of Barra del Colorado

Table 2. Environmental data from positive eDNA samples of *Pristis pristis* detected in Costa Rica. Environmental parameters recorded at each site and station included water flow (Flow; qualitative measure of the body of water sampled), turbidity (Turb.) bottom water temperature (Temp.), and salinity (Sal.). Positive eDNA samples of *P. pristis* were only detected with 20 µm pore size filters. Total filtering time and volume (Vol.) are indicated. An inhabitation test (QSY-IPC) was conducted in all samples analyzed. Inhibition cycle threshold (C_t) difference is shown in the last column

Site/station	Latitude Longitude	Season	Depth (m)	Flow (m)	Turb.	Temp. (°C)	Sal. (ppt)	Pore size (µm)	Filtering time (min)	Vol. (l)	Inhibition C_t diff
Boca San Carlos											
S1	10.78282° N 84.20072° W	Rainy	2	Slow	0.45	29.9	0	20	6	3	0.753
S1	10.78282° N 84.20072° W	Rainy	2	Slow	0.45	29.9	0	20	6	3	0.753
S2	10.78543° N 84.19724° W	Dry	0.3	Fast	0.25	27.6	0	20	5	3	0.503
S2	10.78543° N 84.19724° W	Dry	0.3	Fast	0.25	27.6	0	20	5	3	0.503
S2	10.78543° N 84.19724° W	Dry	0.3	Fast	0.25	27.6	0	20	6	3	0.503
S2	10.78543° N 84.19724° W	Dry	0.3	Fast	0.25	27.6	0	20	5	3.5	0.503
S4	10.78982° N 84.19172° W	Dry	2	Slow	0.7	28	0	20	5	3.2	0.542
S4	10.78982° N 84.19172° W	Dry	2	Slow	0.7	28	0	20	5	3.25	0.542
S4	10.78982° N 84.19172° W	Dry	2	Slow	0.7	28	0	20	6	3.2	0.542
Boca Cureñita											
S1	10.77124° N 84.09329° W	Rainy	1.5	Fast	0.35	28	0	20	8	2	0.092
S2	10.76614° N 84.08883° W	Rainy	1.2	Fast	0.4	28	0	20	8	2	0.149
S4	10.76006° N 84.07448° W	Rainy	3.4	Fast	0.35	28.4	0	20	7	1.5	0.427
S6	10.76593° N 84.06249° W	Dry	9	Fast	0.5	28	0	20	3	3.7	0.383
S7	10.77096° N 84.11118° W	Rainy	1.4	Fast	0.3	27.8	0	20	7	2	0.534
Barra del Colorado (Estuary)											
S1	10.80198° N 83.58608° W	Rainy	1.5	Moderate	0.5	28.2	0.1	20	8	2.8	1.843
S2	10.79511° N 83.58985° W	Rainy	3.4	Fast	0.5	28.4	0.1	20	5	1.5	1.574

3.2. DNA sequences

P. pristis sequences from the San Juan-Colorado River basin showed a single base-pair transition (T to C) of the mitochondrial 12S gene (Table S2). This single base-pair transition appears to be characteristic of the samples from this study, as it is absent from *P. pristis* sequences obtained from Australian and Papua New Guinea tissue samples (M. K. Cooper unpubl. data).

4. DISCUSSION

Understanding the true extent of the distribution of rare and threatened aquatic species is crucial to identifying priority areas for conservation, particularly in freshwater ecosystems where data are scarce and anthropogenic pressures are rising (He et al. 2021, Su et al. 2021, Scherer et al. 2023). This study provides (1) indirect records of the Critically Endangered large-

tooth sawfish *Pristis pristis* in freshwater habitats of Costa Rica, Central America, based on eDNA surveys; (2) strong evidence that the San Juan-Colorado River basin, a natural border between Costa Rica and Nicaragua, is an important region for *P. pristis*; and (3) confirmation that eDNA analysis in fast-flowing, sediment-rich freshwaters is sensitive enough to detect rare aquatic species such as sawfish, which can improve our knowledge of their distribution in poorly studied tropical habitats. Furthermore, based on the protocol developed by Cooper et al. (2021), all amplicons from our study matched *P. pristis*, whereas field controls, extraction negative controls, and qPCR NTCs showed no amplification for sawfish (*P. pristis* and *P. pectinata*) or other related species. Collectively, these findings suggest that our results are robust and add to the growing body of evidence that well-designed eDNA surveys can serve as a rapid, cost-effective tool for identifying important habitats for the conservation of threatened aquatic species in freshwater and coastal habitats (Thomsen et al. 2012, Nguyen et al. 2020, Saenz-Agudelo et al. 2022).

Our eDNA surveys detected *P. pristis* in 3 of the 18 surveyed sites across the entire country. Sites with positive detections were distributed along the San Juan-Colorado River basin, supporting the findings of Valerio-Vargas & Espinoza (2019) that this entire system is likely one of the last remaining areas where *P. pristis* is still present in Costa Rica. This is further supported by the fact that part of the sampling in Boca San Carlos and Boca Cureña-Cureñita was conducted during the dry and rainy season, with positive detections of *P. pristis* in samples from both seasons. Results from the estuary in Barra del Colorado (northern Caribbean) showed that the species is also present in freshwater and brackish habitats near the mouth of the river, which is separated by 60 km from Boca Cureña-Cureñita and 80 km from Boca San Carlos.

The detection of *P. pristis* in the San Juan-Colorado River basin required a field protocol that was sensitive enough to detect traces of DNA from a rare species found in fast-flowing, sediment-rich freshwater. Previous modelling work on the freshwater round goby *Neogobius melanostomus* suggests that eDNA could potentially travel distances of up to 50 km under certain circumstances (Nevers et al. 2020, Seymour et al. 2021), but other studies have shown that DNA degradation is likely to occur over long distances, particularly in warm freshwater habitats (Dejan et al. 2011). In fast-flowing, sediment-rich and unstable environments such as the San Juan-Colorado River basin, the lifespan of eDNA is likely to be hours, suggesting that positive detections of *P. pristis* may be the result of the species moving along this system rather than DNA transport. In the 1970s, a tagging study conducted along the entire San Juan-Colorado River basin showed that *P. pristis* was able to move up to 350 km from Lake Nicaragua to the Caribbean coast (Thorson 1982a). Moreover, there have been multiple catch records of juvenile and adult *P. pristis* (Figs. 3 & 4) along the San Juan-Colorado River since 2015 (Valerio-Vargas & Espinoza 2019, M. Espinoza et al. unpubl. data), providing further evidence that the species may be more abundant in this region than in other areas of Costa Rica.

Positive eDNA detections and recent catch records of *P. pristis* along the San Juan-Colorado River basin raise the possibility that the species is recovering in this region (Figs. 3 & 4). However, even low levels of fishing pressure can have a significant impact on this population, similar to what happened in the 1970s and 1980s (Thorson 1982b, Espinoza et al. 2022). The sawfish in the Western Atlantic subpopulation of Central America face several threats, the most important being net fishing in Lake Nicaragua and Barra del Colorado (Espinoza et al. 2022), despite the fact that current legislation in Costa Rica and Nicaragua protects both species in all or some parts of their range (Espinoza et al. 2022).

Our surveys did not detect *P. pristis* in the southern Caribbean, despite records from 2013 and 2017 indicating the species was present in this region (Valerio-Vargas & Espinoza 2019). In more than 30 yr of ichthyological surveys, sawfish have been absent from Laguna Gandoca (e.g. Chacón-Chaverri 1994, Morera & Brenes 2010), and there is only one confirmed record from the Río Sixaola, near the community of Bonife, <10 km from the river mouth, in the late 1990s (W. McLarney pers. comm.). The Laguna Gandoca has the largest and most developed mangrove forest in the Costa Rican Caribbean and has been a Ramsar site since 1996 (Windevoxhel et al. 1995, Cortés & Wehrtmann 2009). Although this lagoon appears to be a suitable habitat for sawfish, the access and connection to coastal waters through a sandbar is unstable and highly dynamic due to oceanographic and climatological conditions (Cortés & Wehrtmann 2009).

The absence of *P. pristis* from our study and other field surveys conducted in the southern Caribbean of Costa Rica (Chacón-Chaverri 1994, Morera & Brenes 2010) may be explained by (1) the historic low natural abundance of the species in the area (Espinoza et al. 2022), (2) the collapse of its populations in Costa Rica since the 1980s due to overfishing and other threats (Valerio-Vargas & Espinoza 2019), (3) the unstable

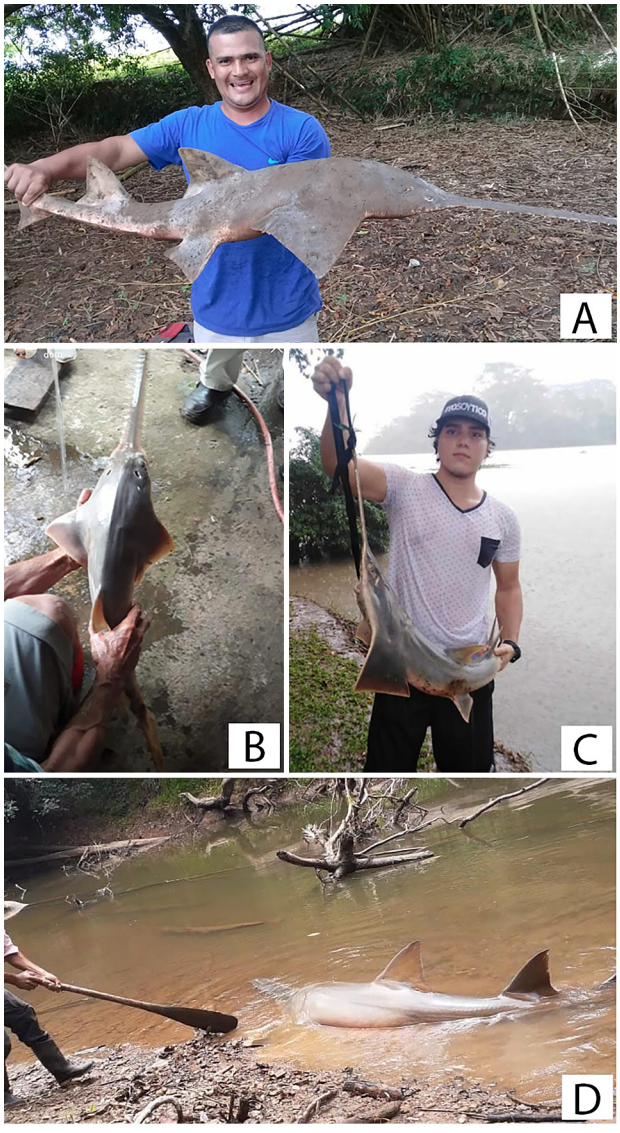

Fig. 3. Recent captures of *Pristis pristis* along the San Juan River. (A) Juvenile (<1.5 m) caught at Remolinito in March 2020 (photo credit Marlon Oporta); (B) newly born (<1 m) caught at Boca San Carlos in September 2020 (photo credit Miguel Su); (C): juvenile (<1.5 m) caught at Boca San Carlos in November 2020 (photo credit Oscar Carranza); (D): adult (2.5–3 m) caught near the Machuca rapids in June 2021 (photo credit Bryant Reyes). All sawfish were released alive and unharmed

accessibility to suitable lagoon habitats along the coast (Sheaves 2005), and (4) the relatively low spatial and temporal resolution of our sampling design. Due to budget constraints, our eDNA surveys only provided a quick snapshot of the distribution of *P. pristis* in areas of Costa Rica that were considered key for the species. Further surveys are needed throughout the Central American Caribbean to refine the search for sawfish in this region.

The HNTS in the southern Pacific region of Costa Rica has been identified in previous studies as an important area for sawfish (Valerio-Vargas & Espinoza 2019). The HNTS is one of the largest and most productive mangrove systems in Central America (Jiménez & Soto 1985, Jiménez 1999), with a relatively large number of contemporary records of *P. pristis* (Valerio-Vargas & Espinoza 2019). However, despite having sampled more of the HNTS than other areas, we were unable to detect *P. pristis* using eDNA analysis. Our Pacific coast surveys included other sites because of their remoteness, conservation status, availability of suitable sawfish habitat, and recent confirmed records of the species (E. van der Poll pers. comm.), but we also failed to detect the presence of *P. pristis* in these areas.

There are significant habitat and environmental differences that may explain why *P. pristis* was present in the San Juan-Colorado River basin but absent from the Pacific coast of Costa Rica (Abell et al. 2008, Cortés & Wehrtmann 2009, Herrera 2016). In the San Juan-Colorado River basin, heavy rainfall year-round provides a continuous supply of fresh water that flows down from Lake Nicaragua (Abell et al. 2008). There are also no mangroves within this system, and the tidal amplitude is relatively low (<50 cm) throughout the Caribbean (Cortés & Wehrtmann 2009). These unique conditions may influence the behavior and ecology of *P. pristis*, restricting its movement to freshwater habitats, with only some individuals migrating downstream to coastal environments from the northern Caribbean coast of Costa Rica (Thorson 1982a,b). In contrast, the Pacific coast of Costa Rica is characterized by marked seasonal and spatial differences in precipitation and freshwater flow (Abell et al. 2008, Cortés & Wehrtmann 2009, Herrera 2016), which are important drivers of seasonal movement and migratory patterns of aquatic species, including sawfish (Simpfendorfer et al. 2011, Lear et al. 2019).

Although there are no studies on seasonal patterns of *P. pristis* within the Eastern Pacific subpopulation, anecdotal evidence suggests that the Gulf of Nicoya and Río Tempisque (one of the largest rivers in the Gulf of Nicoya, central Pacific of Costa Rica) likely served as a historic pupping ground for *P. pristis* during the rainy season (Valerio-Vargas & Espinoza 2019). Except for Río Tempisque, samples from the northern Pacific (e.g. Bahía Thomas, Bahía Santa Elena, Bahía Potrero Grande, Bahía Naranjo, Playa Danta-Dantita) were collected during the rainy season (Herrera 2016). However, the Pacific coast of Costa Rica experienced a severe rainfall deficit in 2019 (IMN 2019a). Samples from the Palo Seco estuary in

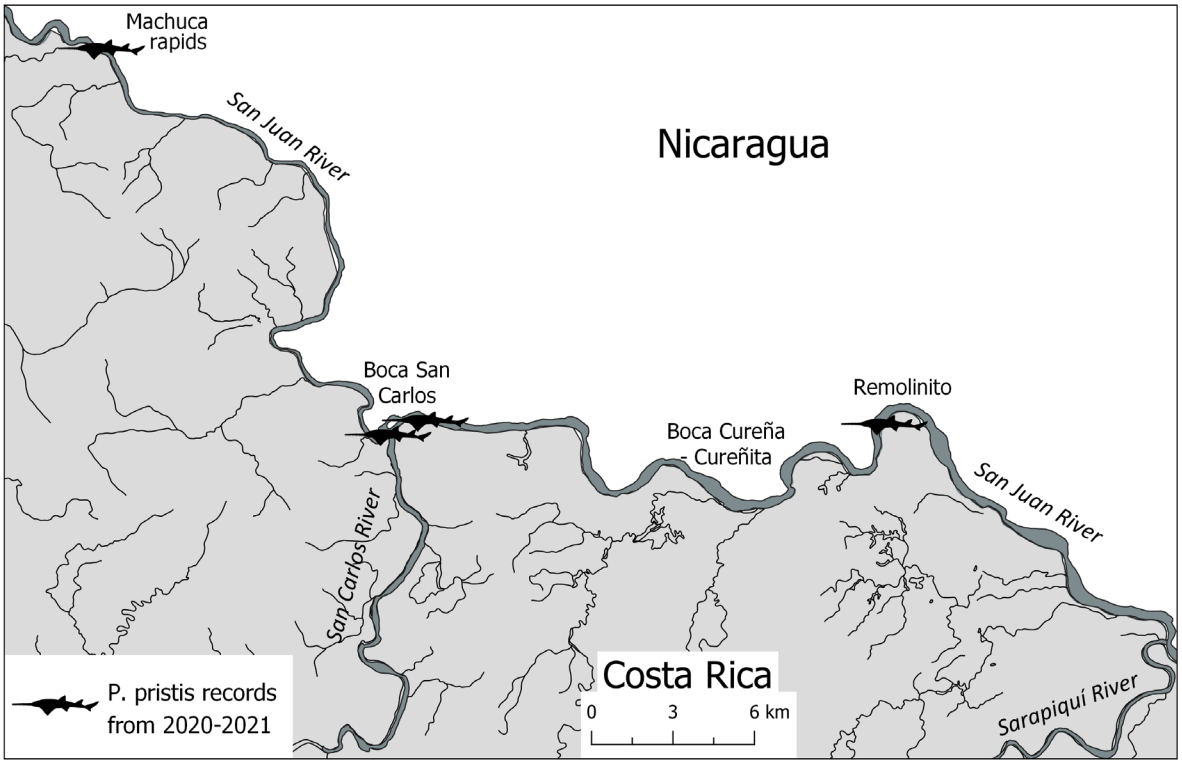

Fig. 4. Location of recent captures of *Pristis pristis* along the San Juan River. Remolinito: juvenile (<1.5 m) caught in March 2020; Boca San Carlos: newly born (<1 m) caught in September 2020 and juvenile (<1.5 m) caught in November 2020; Machuca rapids: adult (2.5–3 m) caught in June 2021. Positive eDNA samples were collected from Boca San Carlos and Boca Cureña-Cureñita

the central Pacific and from the HNTS in the south Pacific were collected during the dry season, while Pejeperro and Pejeperrito coastal lagoons were sampled during a severe rainfall deficit (IMN 2019b). According to Peverell (2010), sawfish from the Indo-West Pacific subpopulation appear to use freshwater flow and rainfall patterns as environmental cues to trigger pupping, which occurs late in the rainy season in the Northern Territory of Australia. As a result, seasonal changes in rainfall and freshwater flow along the Pacific coast of Costa Rica may have influenced the use of coastal, estuarine, and freshwater habitats by sawfish, and thus the detection of the species in our eDNA surveys.

P. pristis is known to reproduce annually in the Indo-West Pacific (Peverell 2010) and biennially in the Western Atlantic (Thorson 1976). This crucial aspect of its life history, for which there are limited data (Kyne et al. 2021), may also have reduced the likelihood of eDNA detection in different environments, as young-of-the-year and pregnant *P. pristis* may have been absent from pupping grounds during our sampling year. Changes in water flow, environmental conditions, microhabitat structure, and sediment load can also affect the likelihood of detecting sawfish if the species is present (Dejean et al. 2011, Bellemain et al. 2016, Cooper et al. 2021). For example, in freshwater field experiments with Siberian sturgeon and Bullfrog tadpoles, DNA is detectable for less than 1 mo after removal of the the species source of DNA (Dejean et al. 2011). High water temperatures and UV radiation can damage and reduce the density of DNA molecules in the environment (Dejean et al. 2011), which means that strong seasonal variations in water temperature, flow, and precipitation in some areas can affect DNA persistence and detection (Stoeckle et al. 2017). In addition, DNA persistence is also influenced by factors other than the environment, including field sampling protocols, sample preservation, and DNA extraction methods (Cooper et al. 2021).

P. pectinata was not detected in any of the samples collected from the northern and southern Caribbean regions of Costa Rica, despite the historical presence of the species in these areas (Thorson 1982a, Valerio-Vargas & Espinoza 2019). The last confirmed record of the species occurred in the southern Caribbean in 2002 (J. Valerio pers. comm.). However, the subtle differ-

ences between sawfish species may limit fishers' ability to distinguish them (Hossain et al. 2015), opening the possibility that some of the records in these areas could have been misidentified (Valerio-Vargas & Espinoza 2019). Following a reduction of ≥95% of its population and 81% of its geographic range (Dulvy et al. 2016, Carlson et al. 2022), it is likely that *P. pectinata* is now restricted to its core distribution range on the southern east coast of the USA and the Bahamas (Brame et al. 2019, Carlson et al. 2022) and is found in low numbers throughout Latin America (Bonfil et al. 2021, 2024), thus limiting our ability to detect the species through eDNA analysis. Further studies on the Caribbean coast of Costa Rica can confirm whether the lack of detection of *P. pectinata* is due to the use of smaller filters (e.g. <20 µm pore size) which failed to detect both species, low survey effort, or the fact that the species is less common or now absent from some areas.

There are few records of *P. pectinata* in Belize and Honduras (Dulvy et al. 2016), but detailed information on the distribution and abundance of this species in Central America is still lacking. Belize and Honduras have some of the highest mangrove cover along the Central American Caribbean coast (Spalding et al. 1997), which is known to be critical habitat as nursery grounds for *P. pectinata* (Simpfendorfer et al. 2011, Guttridge et al. 2015). There are only 2 relatively small and isolated mangrove forests in the Costa Rican Caribbean, one at Moín (central Caribbean), which is heavily affected by coastal development and pollution, and the other at the Laguna Gandoca Wildlife Refuge (southern Caribbean) (Cortés & Wehrtmann 2009). Although historically present in Costa Rican waters, *P. pectinata* was far less abundant than *P. pristis* in the 1960s and 1970s, when Thorson (1982b) recorded hundreds of *P. pristis* and only 16 *P. pectinata* in 14 yr of sampling. The latter were caught in Barra del Colorado and Boca Samay (northern Caribbean of Costa Rica), where there are no mangroves. Furthermore, to our knowledge, there are no reports of juvenile *P. pectinata* in Costa Rican waters. Given their migratory behavior and the relatively low number of confirmed records of *P. pectinata* in Costa Rican waters, it is also possible that some individuals are potential migrants from other countries in Central America, such as Belize and Honduras (Brame et al. 2019).

4.1. Challenges and limitations of our eDNA survey

In an ideal survey design, all sites selected for eDNA analysis should have had a similar sampling effort, including similar sampling replication within stations, similar pore size filters, and a similar volume of water filtered. However, this was not always possible in our study. In some regions we had a higher survey effort (more sites and sampling stations where samples were collected) than in others, making direct comparisons difficult. Our original sampling protocol was designed to survey as much of the country as possible while prioritizing key areas for sawfish previously identified by Valerio-Vargas & Espinoza (2019). Special attention was also given to remote, inaccessible areas with suitable sawfish habitat that had been poorly explored in the past. Ultimately, sampling effort also depended on the accessibility and unique conditions of each site as well as budget constraints.

At most sites, the excess of sediment in the water forced us to use a large pore size filter (20 µm) rather than the smaller pore size filters (5 or 10 µm) that were the first choice for water filtration based on our protocol (Cooper et al. 2021). Even when 20 µm pore size filters were used, excess sediment during filtration often resulted in significantly less volume being filtered than was originally intended (at least 5 l per replicate sample or 25 l per station). While we are aware of these limitations, it is important to highlight the following facts when comparing our results across sites and regions: (1) *P. pristis* was only detected in 2 sites (Boca San Carlos and Boca Cureña-Cureñita) from the northern region and a site near the mouth of Río Colorado (e.g. estuary of Barra del Colorado) in the northern Caribbean, both of which are part of the San Juan-Colorado River basin and are separated by approximately 60 and 80 km, respectively; (2) these 2 regions had fewer surveyed sites and stations than other regions included in our study; (3) *P. pristis* was only detected in samples using 20 µm pore size filters, and the volume of water filtered at these stations was significantly lower than at other stations; (4) *P. pristis* was only detected in samples from relatively shallow water (<4 m depth) filtered near the surface; and (5) *P. pristis* was detected in Boca San Carlos (northern region) in both seasons, further demonstrating that this method can yield positive results despite seasonal changes in water flow and precipitation.

While eDNA degradation rates of aquatic species in the San Juan-Colorado River basin are likely to be fast given the type of environment (fast-flowing, sediment-rich waters) and conditions (warm water temperatures and constant precipitation), multiple positive detections of *P. pristis* demonstrate that this method can still provide robust results. Future studies, however, should consider potential eDNA transport and degradation rates in fast-flowing, sediment-rich tropical waters. This information can improve the

interpretation of eDNA data, as well as refine the identification of critical habitats for threatened species over different spatial and temporal scales. Our study can also guide future eDNA field surveys in terms of site selection, sampling replication (including total volume filtered), and pore size filters used to detect sawfish in high-flow freshwater habitats with excess sediment.

4.2. Conclusions

Using eDNA to detect rare aquatic species across a wide range of habitats is challenging unless the species persists in the environment for long periods of time or is more abundant in some areas than others (Dejean et al. 2011, Nevers et al. 2020, Wood et al. 2020). Our results indicate that *P. pristis* is currently present in the San Juan-Colorado River basin, but our eDNA survey failed to detect the species in other regions of Costa Rica. Although the primers and probes used in this study were designed based on DNA sequences of *P. pristis* from the Indo-West Pacific subpopulation (Cooper et al. 2021), these sequences were able to match and amplify eDNA from the species in the San Juan-Colorado River basin. Our study suggests that the San Juan-Colorado River basin may be a key region for future conservation efforts to restore *P. pristis* in Central America (Fernandez-Carvalho et al. 2014, Espinoza et al. 2022). Although there have been recent records of the species in Central and South America (Mendoza et al. 2017, López-Angarita et al. 2021, Espinoza et al. 2022), since 2015, there is strong evidence based on multiple sightings (Valerio-Vargas & Espinoza 2019, Espinoza et al. 2022) as well as from our current study that the San Juan-Colorado River basin may still support relatively large numbers of *P. pristis*. Wide-ranging aquatic species that move across international boundaries require regional management and conservation measures (Heupel et al. 2015, Pacoureau et al. 2023). There is an urgent need to promote well-designed, coordinated efforts in Central America that can lead to the recovery of sawfish populations (Valerio-Vargas & Espinoza 2019, López-Angarita et al. 2021, Yan et al. 2021); however, the political instability in Nicaragua poses significant challenges to securing these goals. At the moment, all sawfish species are listed as Critically Endangered by the IUCN Red List, but sustained conservation efforts can lead to their recovery (Harry et al. 2024). Therefore, improved international cooperation and agreements between Costa Rica and Nicaragua are essential to reduce the threats to sawfish in Central America and allow for a stable recovery of the species in its historic core of the Western Atlantic subpopulation (Fernandez-Carvalho et al. 2014).

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