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There must be something in the water: Detecting the endangered largetooth sawfish from aquatic eDNA

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Abstract

Currently, the loss of biodiversity is globally prevalent due to anthropogenic activities creating pressures that threaten species across ecosystems. These threats disproportionally affect species as different exposure levels and specific traits cause some species to become more susceptible than others. The Critically Endangered largetooth sawfish (*Pristis pristis*) has experienced major population declines, owing to their large body size and saw-like rostra increasing their susceptibility to capture in fishing gears. It is now uncertain where the populations persist due to their low abundance. Sawfish were historically present in many parts of Madagascar, but their current distribution and abundance is very poorly understood. With the use of environmental DNA (eDNA), a relatively new tool in detecting threatened species, an attempt was made to detect the presence of *P. pristis* in 19 eDNA samples from northwestern Madagascar. This was done through species-specific primers obtained from Simpfendorfer et al (2016) that target the cytochrome c oxidase subunit I (COI). The species-specific primers were found to successfully amplify the targeted region of the positive controls consisting of six rostrum cartilage samples, as well as fail to align with COI sequences of Malagasy freshwater fish species gathered through GenBank and on-site fin clips, which acted as a negative control. When the specific primers were applied to the 19 eDNA samples, it was found that no *P. pristis* DNA was detected in any of the samples five repeats via the conventional PCR (standard PCR) method, but did detect *P. pristis* DNA within three eDNA samples obtained from an aquarium tank known to contain a *P. pristis* individual. Quantitative PCRs (qPCR) were also performed and resulted in two of samples obtained in Madagascar and the three aquarium eDNA samples indicating the presence of *P. pristis*. Nevertheless, after applying a stringent threshold to ensure the reliability of the results it was concluded that overall *P. pristis* is absent from the environment sampled in northwestern Madagascar. These results reveal that a more thorough sampling effort of the region is needed to provide better estimates of *P. pristis* population status and locations of any remnant populations. Along with demonstrating that eDNA is a powerful tool for detecting *P. pristis,* it also has great potential for informing management and conservation actions for *P. pristis,* especially when coupled with traditional monitoring.

Key words: *Pristis pristis,* Threatened species, Madagascar, Survey technique

Introduction

The current rate of extinction due to human influence is a major concern for the future functioning of ecosystems as well as humanity's well-being (Johnson et al. 2017). Biodiversity loss is experienced globally and affects all ecosystems, such as marine and freshwater ecosystems (Collen et al. 2014, Costello et al. 2017, Johnson et al. 2017). The main threats have been, and continue to be, overexploitation, habitat loss, fragmentation, invasive species, pollution and climate change (Arthington et al. 2016). However, extinctions linked to anthropogenic drivers are not random, as some species are more susceptible to these pressures than others, mainly due to varying life-history traits (Brose et al. 2017). Traits that predispose species to extinction include large body size, low reproductive output and late maturity (Arthington et al. 2016, Brose et al. 2017, Costello et al. 2017, He et al. 2019,

Sievers et al. 2019). Megafauna populations are more vulnerable to overharvesting, due to their increased catchability (Arthington et al. 2016, Giglio et al. 2014) and their tendency to have large home ranges, which increases the threats these species are exposed to. In addition, slower life histories, such as late maturity and low reproductive output, reduce their population recovery rate (He et al. 2019, Hensel et al. 2018, Reis-Filho et al. 2016).

One charismatic megafauna group that is vulnerable to population declines are the sawfishes. These fishes belong to an order of rays, Rhinopristiformes, and are easily recognisable due to their saw-like rostra. Sawfishes are primarily found in coastal marine and estuarine areas due to their adaptability to varying salinities (Faria et al. 2013). Their physiological plasticity to salinity and temperature allows them access freshwater, marine and brackish water habitats, including mangrove forests, which act as important pupping and nursery grounds (Leeney 2017, Whitty et al. 2017).

Sawfishes are large, with adults ranging between two and seven meters long, with the estimates of life-span from nine years (narrow sawfish, *Anoxypristis cuspidata*) to 50 years (green sawfish, *Pristis zijsron*, Simpfendorfer 2013). It is due to these life-history traits that they are extremely vulnerable to population decline, as not only does their large body size increases overfishing pressures but their rostrum is prone to line and net entanglement (Harrison & Dulvy 2014 Poulakis & Grubbs 2019). Other pressures on sawfishes populations include the growing demand for shark fins as well as their predictable migrations between freshwater and marine ecosystems heightening their catchability and increased exposure to human influences (Arthington et al. 2016, Leeney & Poncelet 2015, Poulakis & Grubbs 2019).

Historically, sawfishes were widely distributed and occurred in the coastal regions of 90 countries (Dulvy et al. 2016). However, they are now thought to be the most threatened marine animals in the world (Chowdhury et al. 2018, Hossain et al. 2014), with three of the five species are listed as Critically Endangered: the smalltooth (*P. pectinata*), largetooth (*P. pristis*) and green sawfish, and the other two species listed as Endangered: the narrow and dwarf sawfish (*P. clavata*). This drastic reduction in populations has likely had a large effect on ecological processes as sawfishes are top predators and their loss may ultimately decrease the resistance of whole communities (Brose et al. 2017, He et al. 2019). However, the full effects of their disappearance are not fully understood given the lack of knowledge on the role of sawfishes in the diverse ecosystems they inhabit (Lynam et al. 2017, Pimiento et al. 2017, Poulakis & Grubbs 2019). To fully gauge the pressures confronting sawfishes and start forming potential recovery strategies, the remaining extant range of their populations needs to be determined (Simpfendorfer et al. 2016), although this is a challenging task due to their rarity and tendency to occur in remote areas (Le Port et al. 2018).

Currently, the main methods of determining the distribution of sawfish are through traditional monitoring techniques, which involve the use of archive data, questionnaires and interviews with specialists, traders and fishermen (Chowdhury et al. 2018, Dulvy et al. 2014, Hossain et al. 2014), as well as landing site surveys (Brame et al. 2019). These traditional methods are a cost and time-effective means to assessing the occurrence of the easily identifiable species (Leeney et al. 2018). Nonetheless, traditional monitoring techniques are ineffective for low species abundances and associated with biases (Eiler et al. 2018, Evan et

al. 2017, Le Port et al. 2018), thus providing a limited knowledge of sawfish distributions. Therefore, to improve occurrence data for not only sawfish, but other rare and elusive species, a new technique, called environmental DNA (eDNA) is increasingly utilised to track and monitor them (Biggs et al. 2015, Ma et al. 2016, Welts et al. 2017). Briefly, environmental DNA involves obtaining samples from the environment such as air, soil or water, and extracting any DNA that is present due to shed skin cells, urine, faeces, scales, eggs, larvae, or dead and decomposing individuals (Deiner et al. 2015, Harper et al. 2018, Jo et al. 2017). From these DNA fragments it can then be determined whether one or more target species have been present in the sampled environment without direct visual or acoustic confirmation. This greatly reduces any disturbance experienced by species (Boussarie et al. 2018, Davy et al. 2015, Rees et al. 2014, Thomsen & Willerslev 2015). This method has also shown to have equal or a higher sensitivity to species detection than traditional techniques. For example, Schmelzle & Kinziger (2016) found that using eDNA as a tool to detect the endangered tidewater goby (*Eucyclogobins newberryi*) had nearly double the probability of detection than seining, the traditional method of monitoring this species. However, sensitivity does vary over space and time, due to the target species and environmental factors that affect the persistence, abundance and distribution of DNA in the environment (Furlan et al. 2016, Rose et al. 2019). These factors include water currents, salinity, sediment type, tidal patterns, concentration of bacteria and fungi and the rate at which each species sheds its cells (Goldberg et al. 2015, Rose et al. 2019, Thomas et al. 2019). In addition, particularly in riverine systems, where sawfishes can be found, the downstream transport affects the spatial distribution of eDNA (Roussel et al. 2015, Stoeckle et al. 2017).

There are also various other considerations when determining the likelihood of species detection, such as whether the eDNA sampling captures a particular phenological event (such as spawning), or how active an organism is (Bracken et al. 2018). Notably, the variation in eDNA persistence can produce false negatives and/or false positives for the absence and presence of the targeted species (Guillera-Arroita et al. 2017, Roussel et al. 2015). False negatives occur when a species is thought to be absent due to the eDNA fragments degrading quickly in a particular environment, or when the collected sample did not capture the target eDNA by chance, as well as the possibility that inhibitors bind to the fragments and affect PCR detection (Guillera-Arroita et al. 2017). In contrast, false positives can occur when target eDNA fragments are transported by a vector in to the environment (e.g. through a predator's faeces), or when a species is wrongly marked as present due to DNA fragments persisting longer in the environment than the species (Bylemans et al. 2018, Guillera-Arroita et al. 2017, Weltz et al. 2017). However, the risk of these occurring can be accounted for in experimental design, through repeat sampling and DNA kits that remove PCR inhibitors such as tannins (Díaz-Ferguson & Moyer 2014, Deiner et al. 2015). Risks can also be minimised by combining traditional techniques with eDNA samples. This can determine the effectiveness of eDNA and plays a part in accounting for false positives and negatives (Rose et al. 2019). The choice of molecular techniques also affects the chances of false positives and negatives occurring, as found for conventional PCR (standard PCR), which is more prone to false negatives in comparison to the quantitative PCR (qPCR) method, due to standard PCR being less sensitive to low DNA concentrations (Ma et al. 2016, Piggott 2016, Wei et al. 2018,

Wood et al. 2019). However, due to qPCR being a more sensitive method it becomes prone to false positives, along with being more costly and less robust that the traditional standard PCR method (Davison et al. 2019, De Ventura et al. 2017). Therefore, the choice of PCR strategies must be considered for the context of the study, as target species abundance and budget affect this decision.

Within the context of sawfish, eDNA has great potential to add to the global conservation efforts of the family, with some studies already been conducted (Lehman et al. 2019, Simpfendorfer et al. 2016). For example, Simpfendorfer et al (2016) working in Northern Australia, designed a set of species-specific primers targeting a 145 bp section of the COI gene of the largetooth sawfish. These primers were tested against eDNA samples taken from three different aquarium tanks, where only one contained a *P. pristis* individual, and it was found that the primers positively identified *P. pristis* in the correct tank. They also detected *P. pristis* in seven out of eight field eDNA samples from a river where *P. pristis* is known to occur. Where the field sample in which *P. pristis* was not detected was suggested to be caused by the it being obtained from the main river channel where eDNA turnover rates are higher (Simpfendorfer et al. 2016).

Historically, sawfishes inhabited much of the African continents eastern and western coastlines (Dulvy et al. 2016). It is now unclear whether they persist at all in West Africa (Leeney & Poncelet 2015, Leeney 2017, Leeney & Downing 2016), whilst several remnant populations are thought to exist in the western Indian Ocean (Leeney 2017, Elhassan 2018, Leeney & Adouhouri in rev.). As their population declines have gone largely unregulated and unmonitored (Dulvy et al. 2016, Humber et al. 2015). For instance, there is little documentation on the *P. pristis* populations of Madagascar, it is only known that populations have been heavily exploited since the early 20th century, primarily for their fins due to their high market price, but also for local consumption (Humber et al. 2015, Le Manach et al. 2011). Hence, local populations are undoubtedly at low numbers. Sudan is one of the only Eastern African countries to have current documentation on current sawfish populations (Elhassan 2018). There is a great urgency to reveal and protect populations that may be left (Poulakis & Grubbs 2019).

The aim of this project was to utilise the methodology of Simpfendorfer et al (2016) and use eDNA to detect *P. pristis* in the riverine and mangrove areas of north-western Madagascar. There is little information on the current distribution of the largetooth sawfish in Madagascar. Thus, through eDNA sampling at three estuaries (Bombetoka, Ambodimadiro, and Mahajamba bay) in the Boeny region, I attempted to determine the presence or absence of *P. pristis* in the environment, with the intention of contributing any findings to informing the protection and recovery of the largetooth sawfish.

Methods

General approach

Before beginning the detection of *Pristis pristis* in the eDNA samples collected in Madagascar with the species- specific primers designed by Simpfendorfer et al (2016): PZ-COI F1: 5'- CCT CCT TCT ACT AGC CTC TGC C-3' and PZ- COI R1: 5'-GGA AGA GATA CCA GCT AAG TGC AA-3'.

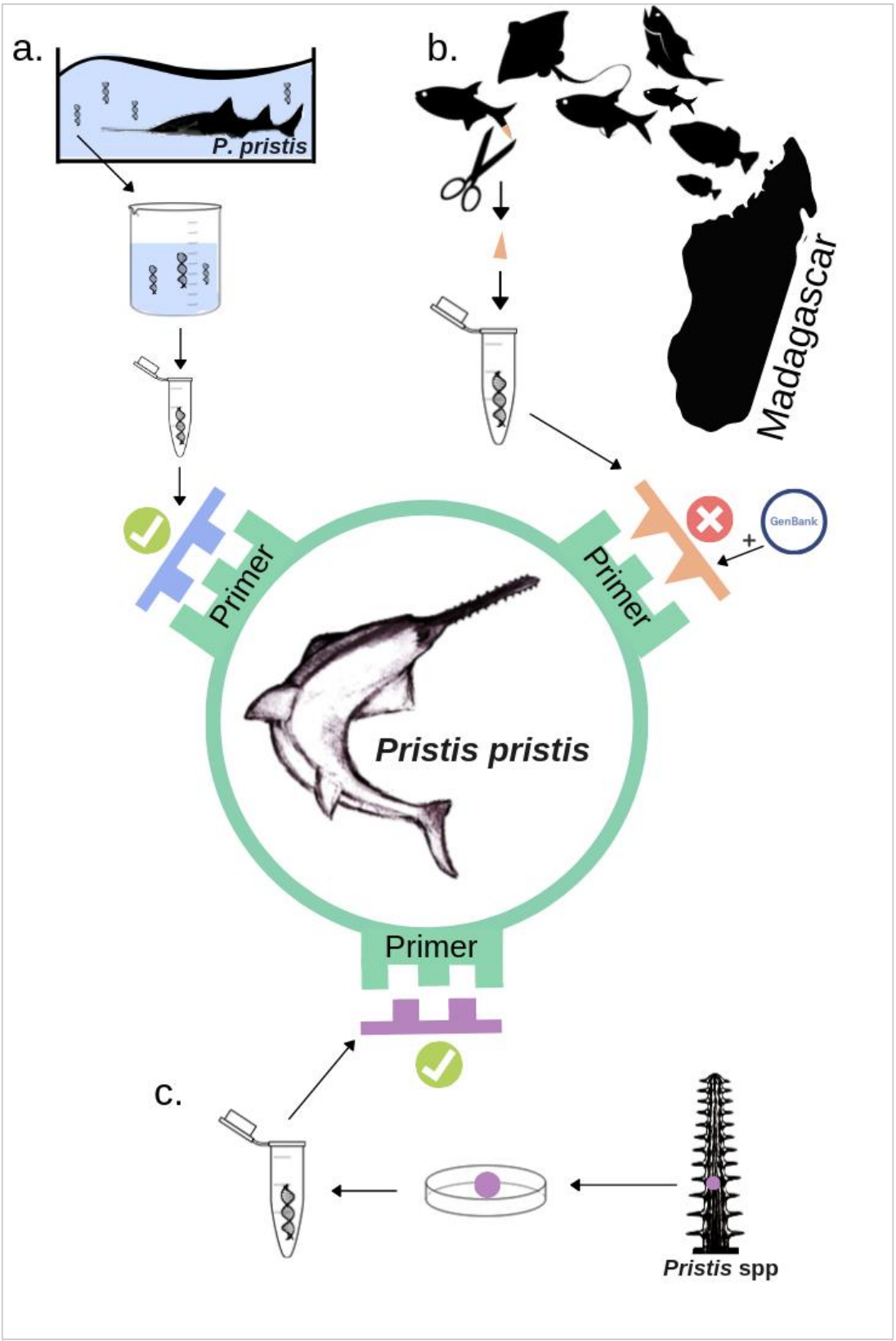

Figure 1. Illustrates the three ways (**a, b & c**) in which the *Pristis pristis* specific primers were tested. (**a**) displays how the primers are expected to pick up *P. pristis* DNA, from an eDNA sample taken from an aquarium tank containing a *P. pristis.* (**b**) illustrates that DNA from the library of COI sequences of Madagascan freshwater fish and elasmobranchs will not align to the specific primers and (**c**) depicts that DNA taken from the cartilage of rostrums are expected to align with the *P. pristis* primers.

The following steps were performed. Firstly, a library of COI sequences was created of fish species that occur in the sampled region (Table 1). This was done in order to check that theSimpfendorfer et al (2016) primers would not align with the COI sequences of other species present in the system and result in the false detection of *P. pristis* (Fig. 1b). Secondly, the Simpfendorfer et al (2016) primers were used to amplify the extracted DNA of six cartilage samples of sawfish collected between the 1940's and 1952 (*P. pristis* and *P. zijsron*) to create positive controls (Fig. 1c). Thirdly, DNA was extracted from the eDNA samples obtained from Madagascar and the Océanopolis Aquarium in France, and lastly, the DNA extracted from these eDNA samples was amplified using both traditional PCR and qPCR methods. From these steps, the detection of *P. pristis* in the samples collected in Madagascar was then possible.

Species list and COI alignment

A species list was created containing fish species that occur in the same region as the sampled site, for each species the COI sequence was obtained, the sequences were used to test whether Simpfendofer et al (2016) primers would amplify any DNA other than *P. pristis* DNA (Fig. 1b). The COI sequences were gathered from two sources; the first source was from DNA extracted from the 18 fin clips collected in the field, which were extracted following a standard DNeasy blood and tissue kit protocol. A partial section of the COI gene was isolated using universal COI fish primers jgHCO2198 and jgLCO1490 designed by Geller et al (2013) and following PCR conditions of 95°C for 2 minutes, with 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 45 s and the final extension of 72°C for 5 minutes. The amplified product was then run for 1 hour through a 1% agarose gel for electrophoresis at 120 V. Bands at the target size of between 600-650 bp were cut out and extracted using Bioflux, sent to the Central Analysis Facility (CAF) in Stellenbosch University for sequencing. The received sequences were added to the sequence database. The second source of sequences were collected through downloading available COI sequences from GenBank of Malagasy freshwater fish species. These species were found in the latest IUCN review on the status and distribution of Madagascar's freshwater biodiversity by Ravelomanana et al (2018). In addition to this, seven COI regions of the elasmobranch species also occurring in the tank of the Océanopolis Aquarium were added to the library. This resulted in over 100 different COI sequences (Table 1). These sequences were then aligned to the Simpfendorfer et al (2016) primers in Geneious Prime 2019.2 (https://www.geneious.com) to find whether the species-specific primers would in theory amplify the non-target COI sequences

Table 1. Lists the names of all the freshwater fish and elasmobranch species gathered from on-site fin clips, available COI sequences from GenBank of species from Madagascan freshwater biodiversity IUCN review by Ravelomanana et al (2018) and the seven elasmobranchs also found in the sampled aquarium to create a library of COI sequences.

Species of the COI library		
"ambamba" - local name	*Gerres filamentosus*	*Paretroplus maromandia*
"tsiangala fitolo" - local name	*Giuris margaritacea*	*Pateobatis jenkinsii*
Acanthopagrus berda	*Glossogobius ankaranensis*	*Pellona ditchela*
Acanthopagrus nebulosus	*Glossogobuis callidus*	*Penaeus monodon*
Acentrogobius audax	*Glossogobuis giuris*	*Pomadasys hasta*
Aetobatus ocellatus	*Hippichthys cyanospilos*	*Psammogobius biocellatus*
Agonostomus catalai	*Hippichthys spicifer*	*Psammoperca waigiensis*
Ambassis ambassis	*Istigobius ornatus*	*Ptychochromis grandidieri*
Amblygaster sirm	*Kuhila sauvagii*	*Ptychochromis makira*
Argyrozona argyrozona	*Kuhlia rupestris*	*Ptychochromis oligacanthus*
Arius madagascariensis	*Leiognathus equulus*	*Ptychochromis mainty*
Aurigenquula fasciata	*Lutjanus argentimaculatus*	*Ptychochromodies betsileanus*
Awaous aeneofuscus	*Lutjanus ehrenbergii*	*Ptychochromodies inornatus*
Bathygobius fuscus	*Megalops cyprinoides*	*Ptychochromodies itasy*
Bedotia geayi	*Microphis brachyurus*	*Ptychochromoides vondrozo*
Bedotia longianalis	*Monodactylus argenteus*	*Ratsirakia legendrei*
Bedotia madagascariensis	*Monodactylus falciformis*	*Rheocles derhami*
Bedotia marojejy	*Moolgarda perusii*	*Rheocles lateralis*
Bedotia masoala	*Nebrius ferrugineus*	*Rheocles wrightae*
Bedotia leucopteron	*Oligolepis acutipennis*	*Rhina ancylostoma*
Butis butis	*Ophiocara porocephala*	*Rhizoprionodon acutus*
Caranx caninus	*Otolithes cuvieri*	*Secutor insidiator*
Carcharhinus amblyrhynchos	*Oxylapia polli*	*Stegosoma fasciatum*
Carcharhinus melanopterus	*Pachypanchax arnoulti*	*Stenogobius polyzona*
Chanos chanos	*Pachypanchax playfairii*	*Teramulus waterloti*
Chirocentrus dorab	*Pachypanchax sakaramyi*	*Terapon jarbua*
Crenimugil crenilabis	*Paratilapia polleni*	*Trachinotus blochii*
Drepane africana	*Paretroplus dambabe*	*Triaenodon obesus*
Elecotris melanosoma	*Paretroplus damii*	*Typhleotris madagascarierisis*
Eleotris fusca	*Paretroplus kieneri*	*Typhleotris mararybe*
Elops machnata	*Paretroplus maculatus*	*Typhleotris pauliani*
Epinephelus bleekeri	*Paretroplus menarambo*	*Valamugil buchanani*
Equulites leuciscus	*Paretroplus nourissati*	*Valamugil robustus*
Favonigobius reichei	*Paretroplus polyactis*	*Yirrkala tenuis*
Gazza minuta	*Paretroplus tsimoly*	

Cartilage positive control

Cartilage shavings were obtained from the rostra of six sawfish specimens collected from the Oceanographic Research Institute (ORI) in Durban, South Africa. Three of the rostrums sampled were of *P. pristis* and the other three were of *P. zijron*. Two of the six specimens are known to be dated to 67 and 79 years ago and three are said to be caught in Durban or Mozambique, with the other three specimens having unknown catch locations. DNA was extracted from the cartilage shavings using the E.Z.N.A. (OMEGA bio-tek) extraction kit following the standard protocol, with the exception of the last elution step. As in the last elution step the buffer was left to sit for two hours compared to the two minutes instructed in the standard protocol, done to ensure maximum DNA yield. The *P. pristis* specific primers

designed by Simpfendorfer et al (2016) were then used to amplify the extracted DNA (Fig. 1c). The PCR conditions of Simpfendorfer et al (2016) were also followed: 95°C for 5 minutes then 35 cycles of 94°C for 30 seconds (s), 60°C for 90 s and 72°C for 30 s, with a final extension at 72°C for 10 minutes. The amplified product was then run through a 2% agarose gel for electrophoresis at 80 Volts (V) for three hours. The targeted 145 bp bands were cut out and extracted using Bioflux-biospin extraction kit, and sent for sequencing at CAF. The received sequences were trimmed and aligned in Geneious Prime 2019.2 to COI sequences of the two sawfish species (*P. pristis* and *P. zijsron* downloaded from GenBank) to ensure the correct region was amplified. In addition the trimmed sequences were Blast searched to GenBank to confirm the identity of the sequences. The identity of theses sequences were only confirmed through having ≥ 97% similarity to the targeted COI region, which was set as a threshold to ensure the sequences were identified to a species level (Andruszkiewicz et al. 2017).

Extraction of eDNA samples taken from Madagascar and the aquarium

A total of 19 eDNA samples were collected from three sites in Northern Madagascar (Fig. 2) in late September to early October of 2018, by Ruth Leeney from the Protect Africa's Sawfishes Project. The samples were gathered mostly from mangrove creeks or alongside mangrove habitats, with one obtained in a rocky bay. Three eDNA samples were also obtained from an aquarium tank in the Océanopolis Aquarium in France (Fig. 1a), which contained a *P. pristis* individual and multiple other species. Both the Madagascar and aquarium eDNA samples were collected by removing one litre of water from the environment, which was then pushed through a 0.22 micrometre sterivex filter using a sterile syringe. The filtering of the water took place within ten minutes to two and a half hours after the water was removed from the environment. Field blanks were also performed at each site by pushing one litre of bottled water through a sterivex filter to be used as a control for field contamination. The filters were stored in an ATL preservation buffer, as suggested to be the optimal approach for yield and detection sensitivity (Spens et al. 2017). They were then transported to Stellenbosch University for later extraction.

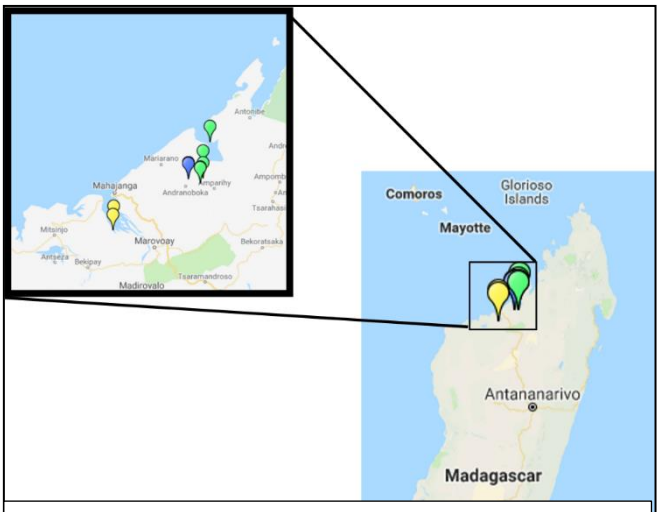

Figure 2. Displays the sample sites in Madagascar. The yellow tags mark Bombetoka Bay, green tags indicate Mahajamba bay and the blue tag marks Ambodimadiro sampling

All extractions of eDNA samples took place in an environmental DNA laboratory (clean room) to avoid any contamination of the samples. Filtered pipette tips, disposable gloves and hazmat suits were used and with the benches and equipment were treated with UV and 10% bleach to reduce contamination risk further. All 22 field and aquarium samples were extracted using a DNeasy Blood and Tissue kit recommended by Deiner et al (2015) for targeting the COI gene of eukaryotes. This protocol was optimised by colleagues at Stellenbosch University for extracting marine eDNA (Czachur et al. in prep). In addition, laboratory blanks of distilled water following the same extraction protocol were produced to indicate any laboratory contaminations. It should be noted that the eDNA samples collected from Madagascar were muddy before extraction, as they all contained fine clay particles within the ATL buffer they were stored in.

Amplification of COI region in the Madagascar and aquarium eDNA samples

With the confirmation that the Simpfendorfer et al (2016) primers were amplifying the targeted region of the *P. pristis* gene and no other species within the sequence library, the primers were then applied to the eDNA samples to detect if *P. pristis* DNA is present. Two PCR strategies were taken, the first was the conventional PCR (standard PCR) method to replicate the approach used in Simpfendorfer et al (2016) and the second method was a quantitative PCR (qPCR) method, due to its higher detection sensitivity of low-copy DNA(Hunter et al. 2015, Ma et al. 2016, Piggott 2016).

Conventional PCR: To increase the chances of *P. pristis* detection, five replicates were performed for each eDNA sample (Piggott 2016) and field and laboratory blanks. For each sample 0.5 µl of 10 µM forward and reverse primers designed by Simpfendorfer et al (2016), 2.5 µl of 10x PCR buffer and dNTP's, 2 µl of Mg+, 0.1 µl Taq and 2 µl of the extracted sample was used, for a 25 µl total reaction volume. PCR blanks containing the same reactants as each sample, except for the 2 µl DNA template which was replaced with 2 µl of nuclease-free water, accompanied with each experiment set up. PCR conditions matched the conditions found in Simpfendorfer et al (2016): 95˚C for 5 minutes followed by 35 cycles of 94˚C for 30 s, 60˚C for 90 s and 72˚C for 30 s, with a final extension at 72˚C for 10 minutes. 10 ul of each sample was then run through agarose gel (2%) for 3 hours at 80 V for electrophoresis. The presence of *P. pristis* DNA in samples was determined by the visualisation of bands near the 150 bp band of the 700 bp low range ladder, with added confirmation from the band size of the positive control of amplified *P. pristis* DNA from the cartilage samples run in each gel. If the band was present it was cut from the gel and extracted with Bioflux and sequenced by CAF. If no band was visualised, *P. pristis* DNA was assumed to be absent from the sample. These sequences were trimmed and then BLAST searched to GenBank. If the percentage of identical sites was ≥ 97% to the *P. pristis* sequence, it was considered confirmation that *P. pristis* DNA was present in the sample.

Quantitative PCR: Similarly, to the standard PCR experiments, five repeats of each eDNA sample, field and laboratory blanks were carried out. However, before running these samples, experiments were run with dilutions of *P. pristis* DNA obtained from the cartilage samples to ensure run method was optimal for detection of low *P. pristis* DNA concentrations. It was found through these experiments that amplification was successful when using the Fast cycling mode taken from the PowerUp ™ SYBR ™ Green Master Mix User

Guide (2016, Pub. no. MAN0013511). However, it was noted that primer dimer formation was prevalent at low DNA concentrations (0.01 ng/µl to 0.0001 ng/µl). This is likely due to the designed primers not meeting the optimal primer guidelines proposed in the User Guide (2016) which suggests they are not optimal due to containing more than two G and C bases in the last five nucleotides at the 3' end of the forward sequence. To eliminate the dimers an additional step was added to the PCR run that increased the temperature of the reaction (to 82°C) past the melting point of the primer dimers (±72°C, determined though melt curves), where detection was then conducted (Blaschke et al. 2000, Bong et al. 2006). The addition of this PCR step resulted in successful amplification of the target region and elimination of primer dimer formation. The method was then applied to the eDNA samples, as it is optimised the detection of *P. pristis* at low DNA concentrations. The eDNA samples were set up in a 96 well plate within a laminar flow UV hood after all surfaces and pipettes were wiped down with 10% bleach and exposed to 15 minutes of UV. Each experiment contained triplicates of six eDNA samples, eDNA aquarium positive controls and non-template controls (NTCs). The samples were set up spatially with filter tips to prevent contamination through aerosols. If any NTCs displayed contamination, the results were discarded. Each reaction consisted of 5 µl of PowerUp SYBR Green Master Mix (2X), 1 µl each of 2.5 µM forward and reverse primer, 2 µl of Nuclease-Free Water and 1 µl of DNA template or 1 µl of Nuclease-Free Water for NTCs. The cycling conditions were as follows: 40 cycles of the three steps of 95°C for 3 s, 60°C for 30 s and 82°C for 40 s (detection stage), with the addition of a Melt curve stage of 95°C for 15 s, 60°C for 60 s and 95°C for 15 s. The melt curve was produced to infer the temperature in which double stranded amplicons are denatured, which was visualised through StepOne software v2.2. From the melt curves of each reaction the presence of the targeted amplicon could be determined, due to the specific melting range of the target sequence. This range was obtained from the melting temperatures (Tm) of 80 positive samples (cartilage and aquarium samples) with a final range between 82.15°C and 82.9°C, and an average Tm of 82.59°C. Therefore, if the product of any of the eDNA sample repeats were shown to melt within this range, it was considered the targeted sequence (Bylemans et al 2018, De Souza et al. 2016). To confirm this further, all samples that denatured in this range were run through 2% agarose gel at 80 V for three hours of electrophoresis and visualised bands were cut out, purified and sent to CAF for sequencing. These sequences were trimmed and then BLAST searched to GenBank. If the percentage of identical sites was ≥ 97% to the *P. pristis* sequence, it was considered a final confirmation that *P. pristis* DNA was present in the sample.

Data analysis

A threshold for a positive result was determined to prevent false positives from occurring, even though it reduces the detection sensitivity of *P. pristis,* because if there is no guideline for eDNA analysis it may cause the study be non-reproducible (Goldberg et al. 2016, Guillera-Arroita et al. 2017). Therefore, to establish consistency the stringent threshold in the study by Harper et al (2018) was applied. This consisted of ≥4/12 repeats being positive to conclude the presence of the target species *Triturus cristatus* in the environment (Harper et al. 2018). Thus, the ratio of this threshold was applied: such that if two of five repeats (considering qPCR and standard PCR repeats separately) should indicate a positive result, the presence of *P. pristis* in the environment could then be inferred.

Results

Species list and COI alignment

It was found that Simpfendorfer et al (2016) species-specific primers failed to align to any of the 107 fish COI sequences collected. This suggests that the presence of the DNA of any of these species within the collected eDNA samples should not affect the detection of *Pristis pristis*. As it is unlikely that the primers would amplify their non-target DNA to produce a false positive.

Positive controls

All cartilage samples successfully aligned with the region targeted by Simpfendorfer et al (2016) species-specific primers (Table 2). However, unlike in Simpfendorfer et al (2016), which found that primers amplified specimens from the genus *Pristis*, with weak product from *P. clavata*, it was found the primers were only capable of amplifying *P. pristis* and *P. zijron* DNA. This was realized through separately aligning the target gene region and primers to the COI region of all five sawfish species and resulted in alignment only being found in *P. pristis* and *P. zijron.*

Detecting *P. pristis*

Conventional PCR: All eDNA samples taken in Madagascar, along with field, laboratory and PCR blanks displayed no target bands on the agarose gel. This suggests *P. pristis* was absent from these samples (Table 3). In the case of the aquarium eDNA samples, it was found all three samples positively indicated the presence of *P. pristis* DNA. However, a false negative was noted for the aquarium sample, BR1, as only four out of five repeats were found to be positive (Table 3).

Quantitative PCR: Due to qPCR having higher sensitivity, contamination was problematic, with multiple experiments having to be discarded and therefore reducing the number of repeats that could be done. The results of the non-contaminated experiments (Table 3) found that two eDNA samples (BA4, B2A) indicated amplified product with the Tm of 82.29°C and once sequenced, the samples produced sequences with over 97% similarity with the targeted region of *P. pristis* COI gene (Table 2). This was only found for one of the five sample repeats of both BA4 and B2A samples, which is below the threshold applied to determine the presence of *P. pristis*. Other samples were also found to have a Tm within the 82.15°C and 82.9°C range and were also sequenced. However, the resulting sequences either were below 97% similar to the targeted *P. pristis* region, too short to be considered or were the sequence of non-target species (Table 2). Therefore, it was found for all eDNA samples gathered from Madagascar, including the field and laboratory blanks, that *P. pristis* was not detected in the environment. However, in the case of the three aquarium eDNA samples, it was found that the qPCR method detected the presence of *P. pristis* in all repeats (Table 3). However, what should be noted is the differences in sequence length produced between the eDNA samples and the cartilage positive control samples (Table 2). This may be due to DNA degrading in the environment before amplification and affecting the quality of sequences, which has resulted in smaller lengths of the target strand in eDNA samples.

Table 2. Lists the percentage (%) of identical base pairs (bp) of received sequences from cartilage positive controls, aquarium eDNA and Madagascar eDNA samples to the. The length of the sequence that was aligned is also given, along with whether the sequence was determined as positively or negatively indicating the presence of *Pristis pristis* DNA.

	Sample	% of identical base pairs to species	Length of sequence (bp)	Species	Determined presence of *P. pristis* DNA
cartilage positive samples	P1	99.3	147	*P. pristis & P. zijsron*	**Positive**
	P2	99.3	146	*P. pristis & P. zijsron*	**Positive**
	P3	99.3	143	*P. pristis & P. zijsron*	**Positive**
	Z1	100	146	*P. pristis & P. zijsron*	**Positive**
	Z2	99.3	146	*P. pristis & P. zijsron*	**Positive**
	Z3	98.6	146	*P. pristis & P. zijsron*	**Positive**
aquarium eDNA samples	BR1	98.9	89	*P. pristis & P. zijsron*	**Positive**
	BR2	98	100	*P. pristis & P. zijsron*	**Positive**
	BR3	99	97	*P. pristis & P. zijsron*	**Positive**
Madagascar eDNA samples	BB3	87.7	81	*P. pristis & P. zijsron*	Negative
	BA2	78.7	86	*P. pristis & P. zijsron*	Negative
	BA4	100	97	*P. pristis & P. zijsron*	**Positive**
	B2A	99	100	*P. pristis & P. zijsron*	**Positive**
	B2B (1)	96	25	*P. pristis & P. zijsron*	Negative
	B2B (2)	74.5	165	*P. pristis & P. zijsron*	Negative
	B2C	79.5	84	human adenovirus	Negative

Table 3. Position of all 19 environmental samples taken in Madagascar within the channel, in addition to the habitat type and the number of repeats of both PCR strategies that positively detected *Pristis pristis.* With the detection of *P. pristis* indicated with a tick or a cross in the bottom right corner of each sample, in addition to the number of repeats indicating a positive detection for the three aquarium samples taken.

Discussion

This study utilised eDNA sampling as a tool for detecting sawfishes from 19 locations in Madagascar. Broadly, this approach confirms the findings of Simpfendorfer et al (2016), that eDNA is a powerful tool for determining the presence of the largetooth sawfish in natural environments. This approach has the potential to provide a better understanding of *Pristis pristis* current distribution and can contribute to identifying areas of remnant populations where conservation efforts can be prioritized (Furlan et al. 2016, Lehman et al. 2019, Simpfendorfer et al. 2016). The final result of the study, after applying the stringent detection threshold, suggests that *P. pristis* is absent from the environmental samples taken from northwestern Madagascar. This is likely due to *P. pristis* populations predicted to be at very low abundances, as well as their presence in mangrove habitats varying over seasons, due to adult largetooth sawfish to only thought to utilise this habitat during the pupping season (Phillips et al. 2017). Therefore, a larger sampling effort will be needed to detect their presence (Le Manach et al. 2011). Another possibility as to why *P. pristis* was absent from the samples may be that the high particle concentration in the sampled water may have affected the PCR sensitivity, resulting in false negatives (Stoeckle et al. 2017).

Two samples (BA4 and B2A; Table 3) did show some amplification of *P. pristis*, although this was only when no detection threshold was applied. However, this only applied to one qPCR of five for both samples and does not account for the occurrence of false positives and in turn reduces the study's reliability (Guilera-Arroita et al. 2017, Harper et al. 2018, Thomas et al. 2019). False positives can occur at any level of the experiment, for example through field contamination, how eDNA transferred within the environment, cross-contamination among samples and laboratory contamination (Harper et al. 2018). This was accounted for through standard contamination measures such as field blanks, laboratory blanks for extraction and amplification steps, the use of decontaminated equipment and other procedures used as part of laboratory practices to minimize contamination risk (Goldberg et al. 2016). Nonetheless, false positives can still occur and affect the results and detection of *P. pristis*. Therefore, the detection threshold was applied to prevent this and ensure consistency (Davison et al. 2019, Harper et al. 2018).

A way forward for detecting *P. pristis* in Africa

If asked to repeat this study, the re-approaching of multiple factors will be needed in order to increase the reliability and sensitivity of *P. pristis* detection, beginning with the consideration of a sampling method that can increase the probability of detection. Hence, to increase the probability of capturing their DNA, larger volumes of the environment should be collected for each sample (Lopes et al. 2017, Pinfield et al. 2019, Schultz & Lance 2015, Sepulveda et al. 2019). For example, Lopes et al (2017), who found that 60 L samples had 14.7% and 7.9% higher estimated probabilities of detection than in 20 L samples for species with low estimated occupancy in the environment. However, what must be considered with increasing sampling volume firstly is the greater amounts of PCR inhibitors are being collected, though this can be accounted for through DNA extraction methods (Sepulveda et al. 2019). Secondly, filtering larger volumes of water will require different filtering methods, as the current

method of pushing water through the filter with a syringe (Deiner et al. 2015, Sigsgaard et al. 2016) will be too time consuming and labour intensive at such large quantities' (Spens et al. 2017). A more efficient method will be required, such as using a battery-powered pump that pushes the water through the filters, i.e. a peristaltic pump (Hinlo et al. 2017, Lopes et al. 2017, Sepulveda et al. 2019). Another factor which to be considered when sampling, is the sampling site with respect to the main water current, as high flow rates have been reported to affect the dilution and dispersal of DNA in the environment (Jane et al. 2015, Jerde et al. 2016, Simpfendorfer et al. 2016). By focusing sampling at sites outside of the main water current and increasing the sampling volume, the chances of capturing *P. pristis* DNA in the environment will be greatly improved.

Another aspect of this study that needs to be reconsidered is the primers taken from Simpfendorfer et al (2016). It was found that the designed primers are prone to the formation of primer dimers and not only amplify *P. pristis* DNA but *P. zijsron* DNA as well. This factor has significant influence on amplification and detection sensitivity of the PCR method, along with causing issues with species-specific detection when *P. pristis* and *P. zijsron* ranges overlap. This problem could be resolved through designing new *P. pristis* primers that target a more species-specific region and that are not susceptible to primer dimer formation. Potentially, resolving this issue may be possible by designing primers to target larger mitochondria gene fragments (Ma et al. 2016) as they provide a higher taxonomic resolution and have recently been found by Bylemans et al (2018) to have no significant differences in degrading rates in comparison to smaller fragments (Bylemans et al. 2018, Jo et al. 2017). Nevertheless, larger fragments are at lower concentrations in the environment than smaller fragments and tend to have a reduced PCR efficiency, therefore effecting detection sensitivity (Bylemans et al. 2018, Jo et al. 2017). For these reasons mentioned, newly designed primers for *P. pristis* detection should still target small mitochondria fragments, even though the taxonomic resolution will be reduced. Therefore, the greatest way to improve the primers designed by Simpfendorfer et al (2016) will be through ensuring that newly designed primers fall within the guidelines of optimal primers found in the PowerUp ™ SYBR ™ Green Master Mix User Guide (2016, Pub. no. MAN0013511), to eliminate primer dimers.

The final factor of this study that needs to be discussed is the choice of PCR strategy. The strategy that is commonly used in eDNA studies that target rare and low abundant species is the qPCR method. This is due to its greater sensitivity in comparison to the more robust standard PCR method (Hunter et al. 2015, Ma et al. 2016, Piggott 2016, Wei et al. 2018, Wood et al. 2019). This was indicated in the results of this study, as it was found that the qPCR method detected the presence of *P. pristis* in two of the eDNA samples (BA4, B2A), where the standard PCR repeats detected no *P. pristis* DNA. Together with the qPCR method detecting *P. pristis* DNA in all repeats where it is known to be present (BR1), unlike those found for the standard PCR method. However, a recent study by Davison et al (2019) found contradictory results, with qPCR being no more sensitive than standard PCR. De Ventura et al (2017) also found this result and provided reasons that standard PCR is the more reliable method due to it being less prone to false positives and false negatives at low target DNA concentrations. In addition to these factors, standard PCR is less costly, faster and more

robust than qPCR (Davison et al. 2019, De Ventura et al. 2017) and remains as an effective tool for eDNA monitoring, especially when compensated for with large sample volumes (Ma et al. 2016). There are other PCR strategies are also available and are becoming more frequently used. An example is droplet digital PCR (ddPCR) (Baker et al. 2018, Hunter et al. 2018, Pinfield et al. 2019, Poulakis & Grubbs 2019), which has been found to be more sensitive than qPCR, particularly when samples contain inhibitors (Coble et al. 2019, Wood et al. 2019). However, a duplex assay of a ddPCR is triple the cost of a 96 well plate needed for a qPCR experiment (Hamaguchi et al. 2018). From this brief overview of these strategies, it should be noted that all the methods are capable of detecting eDNA and that choosing a PCR strategy depends on the level of sensitivity needed and budget limits. In this particular case, with *P. pristis* estimated to be in low population abundances, a highly sensitive strategy should be prioritised, such as qPCR and ddPCR, and should be coupled with greater sample repeats to increase the results reliability.

Overall, eDNA is a powerful tool, and when coupled with traditional monitoring, can greatly inform management and conservation actions for *P. pristis,* as it can provide estimates of the spatial extent of remnant populations. This can then guide and inform other monitoring methods (field surveys and acoustic tagging) and allow the gathering of more detailed information (Guillera- Arroita et al. 2017, Poulakis & Grubbs 2019), such as more in-depth approximation of Northern Madagascar's population status, habitat use and demography. By improving the eDNA detection protocol, the tool will enable the streamlining of sawfish research and maximise the ability of local populations to recover if conservation actions are enforced.

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Figure 1. Illustrates the three ways (**a, b & c**) in which the *Pristis pristis* specific primers were tested. (**a**) displays how the primers are expected to pick up *P. pristis* DNA, from an eDNA sample taken from an aquarium tank containing a *P. pristis.* (**b**) illustrates that DNA from the library of COI sequences of Madagascan freshwater fish and elasmobranchs will not align to the specific primers and (**c**) depicts that DNA taken from the cartilage of rostrums are expected to align with the *P. pristis* primers.