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ORIGINAL ARTICLE

Development of an environmental DNA assay for detecting multiple shark species involved in human–shark conflicts in Australia

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Abstract

The number of human–shark interactions has increased worldwide during the past decade resulting in injuries and fatalities. In Australia, the white shark (*Carcharodon carcharias*), tiger shark (*Galeocerdo cuvier*), and bull shark (*Carcharhinus leucas*) are responsible for the majority of fatal incidents. On the southeast coast of Australia, monitoring programs currently rely on SMART (Shark-Management-Alert-in-Real-Time) drumlines and mesh nets to catch, tag, and monitor shark movement. However, these methods are laborious, costly, and involve the capture of only a fraction of the total shark population. Here, we develop a multiplex environmental DNA assay capable of detecting all three shark species simultaneously from water samples by targeting conserved but specific mitochondrial sequences that are characteristic of each species. The specificity of the assay was validated by testing for cross-amplification across a range of non-target but co-occurring shark species from eastern Australia. We test the sensitivity of the assay on water samples collected from shark capture events and sites where these shark species are known to frequent, and undertake DNA sequencing on positive samples to confirm species haplotype authenticity. Samples collected from one of these sites also demonstrate that eDNA detections are dependent on shark activity in the area. This assay will allow for rapid detection of DNA from each shark species in water samples, providing a cost-effective alternative for monitoring sharks along the east coast of Australia and potentially elsewhere.

KEYWORDS
bull shark, eDNA, environmental DNA, multispecies assay, tiger shark, white shark

1 | INTRODUCTION

Sharks are critical apex predators, which underpin the trophic structure and functionality of marine ecosystems, yet many shark species globally have suffered major declines in recent decades due to human activities. Subsequently, a quarter of the world's sharks and rays are recognized by the International Union for the Conservation of Nature (IUCN) as threatened or at risk of extinction (Birkmanis et al., 2020; Lawson et al., 2020; Roff et al., 2018). Despite these declines, negative human–shark interactions are

increasing globally (McPhee, 2014). The most problematic shark species include the white shark (*Carcharodon carcharias* Linnaeus 1758), tiger shark (*Galeocerdo cuvier* Péron & Lesueur 1822), and bull shark (*Carcharhinus leucas* Müller & Henle 1839), which are responsible for ~56% of all bites and ~98% of all fatalities over the last three decades globally (McPhee, 2014). These three species co-occur in some temperate and subtropical waters around the world, including the eastern seaboard of Australia, a region recognized as one of the world's shark attack hotspots (Chapman & McPhee, 2016).

While risks to human safety are generally overstated, the increasing frequency in shark attacks has led to public demand for programs aimed at suppressing risks to beachgoers (Colefax et al., 2020; Fraser-Baxter & Medvecky, 2018; West, 2011). These include targeted culling programs, which compromise conservation efforts (Cliff & Dudley, 2011; Gibbs et al., 2020), and non-destructive monitoring programs, which allow for the monitoring of shark movements and collection of critical demographic data (Colefax et al., 2020; Spaet et al., 2020a, 2020b; Tate et al., 2019). The most successful non-lethal monitoring program to date has been the Shark Management Strategy (SMS), which currently operates in eastern Australia and is one of the shark mitigation programs run by the New South Wales Government. Initiated in 2015, the SMS program has adopted a combination of SMART (Shark-Management-Alert-in-Real-Time) drumlines (Tate et al., 2019), acoustic and satellite tagging (Spaet et al., 2020a, 2020b), and drones (Butcher et al., 2019; Colefax et al., 2020) to monitor the movement patterns of individual sharks and communicate reports of near-shore shark visitations to the general public via the purposely built SharkSmart app (www.sharksmart.com.au). As of November 2020, 544 *C. carcharias*, 131 *G. cuvier*, and 92 *C. leucas* have been tagged and tracked, and the biological data from individual sharks continue to provide valuable insights into the demographic structure of coastal populations (Butcher et al., 2019; Colefax et al., 2020; Spaet et al., 2020a). However, the program has already come at significant cost to the State Government with over $16 million invested in a 5-year period and an additional $8 million for 2020/21. While the size of resident *C. leucas* and *G. cuvier* populations remain uncertain, it has been estimated that approximately 2500–6750 *C. carcharias* persist on the Australian east coast (Hillary et al., 2018). These figures suggest the number of SMS program tagged sharks represent only a fraction of the total population, highlighting the need for more innovative and cost-effective methods for the detection and monitoring of problem shark species in eastern Australia.

Environmental DNA (eDNA) technologies are revolutionizing the field of wildlife monitoring, providing unprecedented sensitivity for characterizing species presence through the detection of genetic material that organisms shed or excrete into their surrounding environment. The uptake of this technology has increased dramatically in recent years and has been applied widely to surveys of rare or invasive species in freshwater environments (Bohmann et al., 2014; Deiner et al., 2016; Valentini et al., 2016), as well as marine environments, including large pelagic species, providing new insights into species ecology (Berger et al., 2020; Foote et al., 2012; Sigsgaard et al., 2017; Thomsen et al., 2016). Several studies have demonstrated the utility of eDNA technologies for detecting shark species in both shallow and deep-water environments (Bakker et al., 2017; Boussarie et al., 2018; Truelove et al., 2019). Recent examples of eDNA technologies being used for detecting *C. carcharias* (Lafferty et al., 2018) and *C. leucas* (Schweiss et al., 2020) have recently emerged. However, in regions where there is sympatric occurrence of multiple target shark species, careful consideration needs to be given to the specificity of eDNA assays in order to reliably discriminate between co-occurring species. This is particularly pertinent in eastern Australia where there is significant overlap in seasonality and habitat use of *C. carcharias*, *G. cuvier*, and *C. leucas* (Espinoza et al., 2016; Holmes et al., 2014; Lee et al., 2019; Lipscombe et al., 2020; Spaet et al., 2020a, 2020b).

Here, we report the development of a multispecies eDNA assay for simultaneously detecting target shark species in one of the world's major shark attack hotspots. We demonstrate the utility of our assay for detecting and discriminating between *C. carcharias*, *C. leucas*, and *G. cuvier* in eastern Australia using a combination of *in silico*, laboratory, and field trials. We validate the specificity of the assay by testing for cross-amplification across a range of non-target but co-occurring shark species native to eastern Australia and test the sensitivity of the assay on water samples collected around shark capture events. We then apply the eDNA assay in blind surveys at a known shark visitation hotspot at a seasonal timepoint when all three target species are known to occur, demonstrating the capability of simultaneously detecting these species. This new eDNA multispecies assay has the potential to dramatically enhance shark detection and alerting capabilities in shark attack hotspots such as eastern Australia, providing a rapid, cost-effective, and non-invasive alternative to traditional survey methods. We expect the application of the assay could help reduce risks of shark attack and allow further insights into patterns of species movement, near-shore visitation, and habitat use for informing future conservation management.

2 | METHODS

2.1 | DNA extraction from tissue samples and Sterivex® filters

DNA was extracted from shark tissue samples and Sterivex® 0.22 μM filters using the Qiagen DNeasy Blood & Tissue Kit (using the spin-column protocol), with tissue samples extracted in a separate room from filter samples. For tissue samples, total genomic DNA (gDNA) extractions were performed following the standard Qiagen DNeasy Blood & Tissue Kit protocol and using approximately 10 mg of muscle tissue. DNA extractions from Sterivex® filters were performed by initially adding 540 μl of ATL buffer and 40 μl (10 mg/ml) proteinase K to each filter unit, which was subsequently sealed and incubated at 56°C for 3 h with constant agitation. Next, the lysis solution was transferred into new 2-ml tubes, and extractions were completed following the standard Qiagen DNeasy Blood & Tissue

Kit protocol with the following minor adjustments: 500 µl AL buffer, 500 µl ethanol, and final elution step of 100 µl AE buffer for each sample. Extracted DNA samples were stored at −20°C until required for genetic analysis.

2.2 | eDNA assays development

Environmental DNA assays have previously been developed for *C. carcharias* in California (Lafferty et al., 2018) and *C. leucas* in the Gulf of Mexico (Schweiss et al., 2020). We initially tested these assays to determine their specificity to *C. carcharias*, *C. leucas*, and non-target shark species in our study region of northern New South Wales, Australia. Initial *in silico* analyses of each assay suggested potential compatibility (matching primer and probe sequences) with various non-target shark species native to Australia. Quantitative polymerase chain reaction (qPCR) subsequently confirmed non-target cross-amplification in both assays (see results). We therefore downloaded the complete mitochondrial genome sequences from GenBank (www.ncbi.nlm.nih.gov) for our three target species and seven non-target shark species known from our study region to design species-specific assays. Unique regions were first identified by aligning all genomes in Geneious (vers. 10.2.5; https://www.geneious.com). Once target regions were identified, assays were designed using the custom TaqMan® Assay Design tool (https://www.thermofisher.com/order/custom-genomic-products/tools/cadt/) and primer and probe specificity was checked *in silico* using primerblast (https://www.ncbi.nlm.nih.gov /tools/ primer-blast/) with no non-target cross-amplifications identified. Species-specific TaqMan® copy number assays for each species were ordered from Thermo Fisher Scientific Inc., labeled with FAM (*C. carcharias* and *G. cuvier*) and VIC (*C. leucas*) fluorophores. Once assays had been tested individually on all target and non-target shark tissue samples (see below), we then transferred the *G. cuvier* and *C. leucas* assays to the PrimeTime® qPCR method (Integrated DNA Technologies) labeled with HEX (*G. cuvier*) and Cy5 (*C. leucas*) fluorophores (Table 1) so that we could run all assays in a single multiplex reaction and detect all three fluorophores.

Real-time TaqMan and PrimeTime qPCR assays were conducted using a Roche LightCycler 480 II system in a 384-well format. 10 µl reactions containing 5 µl of KAPA probe force PCR Master Mix (Merck), 0.5 µl of each TaqMan or PrimeTime qPCR Assay, 1.5 µl ddH_2O, and 2 µl of template DNA were prepared in triplicate. Included in each 384-well assay plate were control reactions containing a 10-fold dilution series from 1000 to 1 picogram of gDNA and a negative control with no DNA template. The amplification occurred in conditions of 3 min at 98°C, followed by 10 s at 95°C and 20 s at 60°C for 50 cycles. The amplification profiles of each PCR were used to determine the cycle quantification (Cq) value using the Absolute Quantification module of the LightCycler 480 II software package. A TaqMan Exogenous Internal Positive Control (VIC probe) was run for each sample to test for the presence of PCR inhibitors. No inhibition was detected on any eDNA sample. Quantitative PCRs were undertaken in a separate dedicated room, while environmental DNA extractions were undertaken in a room dedicated to low-quantity DNA (a separate room from where tissue DNA extractions were undertaken). Negative controls were included at all stages (DNA extraction, qPCR) so that contamination issues could be identified if present; however, no contamination was detected. A sample was considered positive if 1/3 qPCR replicates detected the target DNA.

2.3 | Primary efficiency, limit of detection, and quantification

Primer efficiency, limit of detection (LOD), and limit of quantification (LOQ) were assessed following the protocol and curve fitting method described in Klymus et al. (2020). For the standard curve, serial dilutions of gDNA derived from tissue extractions were prepared in elution buffer AE, Qiagen. The 10-fold dilution series spanned over five orders of magnitude, ranging from 1000 to 0.1 picograms with an additional onefold dilution to 0.05 picograms of DNA, measured with a Qubit 2.0 fluorometer (Invitrogen). Each dilution was run with twelve replicates, and reported Cq (cycle quantification) values were used to determine primer efficiency, LOD, and LOQ. Amplification efficiency

TABLE 1 Primers and labeled probe sequences targeting different regions of the mtDNA regions for *C. carcharias*, *G. cuvier*, and *C. leucas*

Species	Common name	Gene region	Amplicon size (bp)	Primer/Probe sequence
C. carcharias	White shark	DLoop	128	Primer 1 5′-CCACTCACAGAGACTTAAGTATATATTATGT–3′
				Primer 2 5′-GTCAGGTTTGATCAGTTGATGAGG–3′
				Probe FAM-ATGAAATGTCATGAGTAGAATAA-NFQ
G. cuvier	Tiger shark	DLoop	92	Primer 1 5′-CATAGGTCCATTTGATATCAACATAAG–3′
				Primer 2 5′-CCTTCTCAACCGATGAATAGTT–3′
				Probe 5′-/5Cy5/TTCGACCTATATTCAATTATCTTCACACCC/3IAbRQSp/–3′
C. leucas	Bull shark	ND5	228	Primer 1 5′-GGGATAGAAAATATTAGGTCATAAAGGTCAAG–3′
				Primer 2 5′-GGGAGGGTTAATGACAGTGTTTT–3′
				5′-/5HEX/ATGCAGCAAA/ZEN/GGTTTAAAA/3IABkFG/–3′

was determined by plotting Cq values against gDNA dilutions and calculating the linear slope and the coefficient of determination (R^2) value.

2.4 | Specificity testing

We tested the specificity of each assay on 10 picograms of gDNA extracted from tissue samples from two individuals for each of 11 shark species native to eastern Australia (captured and provided by the SMS team). The performance of each assay was tested against *C. carcharias*, *C. leucas*, *G. cuvier*, Mako shark (*Isurus oxyrinchus*), Blacktip reef shark (*Carcharhinus melanopterus*), Smooth hammerhead (*Sphyrna zygaena*), Dusky shark (*Carcharhinus obscurus*), Greynurse shark (*Carcharias taurus*), Silky shark (*Carcharhinus falciformis*), Bronze Whaler (*Carcharhinus brachyurus*), and Spinner shark (*Carcharhinus brevipinna*). The qPCRs were performed as outlined above in triplicate for each sample, with appropriate negative controls, and were repeated when two of the assays were changed to PrimeTime® assays with different fluorophores.

2.5 | Field eDNA samples

We validated our eDNA assay with 30 x 1 L water samples collected from sites off the coast of northern NSW (Figure 1). We sampled water adjacent to five captured *G. cuvier* and three *C. carcharias* that were being processed next to a vessel ~500 m from the shore as part of the SMS SMART-drumline program in 2019 and 2020 (Figure 1). On each occasion, the person taking the water samples was not involved in direct shark handling procedures. Two 1 L water samples were collected within 2 m of each captured shark in sterile bottles (taken at a depth of approximately 0.5 m using an extension pole). Samples collected next to a *G. cuvier* capture were labeled T1-10, while samples collected next to a *C. carcharias* were labeled W11-16. From two of the *C. carcharias* captures, we also took two 1L water samples ~250 m away (labeled W17-20) from the site of capture within 15 min of release of the shark. The last samples targeting *C. leucas* were duplicate 1 L water samples collected in sterile bottles on four occasions over a two-week period during May 2020 from the southern end of Lighthouse Beach, Ballina (28.874989 S, 153.592648 E), following positive drone detections (P. Butcher, pers. comm.). Duplicate 1 L water samples were also taken from within 2 m of a VR4G listening station (again taken at a depth of approximately 0.5 m using an extension pole) positioned 500 m offshore at Lighthouse Beach following a recent (<5 min) acoustic detection of a tagged *C. leucas* individual. The VR4G listening stations can detect tagged individuals within a ~500 m radius (Spaet et al., 2020b). Samples that targeted *C. leucas* were labeled B21-30.

Within 5 min of water sample collection, each 1 L water bottle was processed by drawing water into a Hapool 60-ml sterilized disposable syringe (Shandong Hapool Medical Technology) and then pushing the water through an attachable (leur lock) Sterivex® 0.22 μm filter unit (Merck). This process was repeated until 1 L of water had been passed through the filter. Filter units were stored on ice in a dark storage container following collection and shipped to the laboratory for processing. Once at the laboratory, samples were stored at −20°C until DNA extraction.

2.6 | Negative field control samples

We took several negative field controls to help assess the multispecies assays. Firstly, we assessed the potential for false positives occurring during eDNA sampling on the boat. A bottle containing sterile water was opened after a shark capture, and 1 L of water was passed through a Sterivex® filter repeating the process that had been undertaken for eDNA sampling (by the same person who took all eDNA samples). This was conducted on three shark capture occasions (samples labeled C31-33).

Secondly, we sampled twice daily (~8.00 AM and ~5.00 PM) for five consecutive days at a VR4G listening station at Evans Head (within 2 m of the station at a depth of 0.5 m) at the end of the white shark season when shark activity decreases markedly (Spaet et al., 2020b). A total of 10 samples were taken across the 5 days (labeled C34-43). These samples were used to assess background activity (shark DNA) in the area when there are no detections on the VR4G listening station. No sharks were detected by the acoustic station during the 5 days of sampling or for at least seven days prior to sampling.

2.7 | Sequencing positive samples

Six eDNA samples (T1, T2, T4, B22, B25, and B30) that returned a positive result for *G. cuvier* DNA and four eDNA samples (W16, W19, W2, B22, and B25) that returned a positive result for *C. carcharias* DNA were subsequently amplified by PCR using the respective species amplicon primers tagged with M13 tail sequences. Products were then sequenced using Sanger sequencing (ABI 3730xl, Macrogen Korea) in dual directions using M13 primers and compared to reference sequences to confirm species haplotype authenticity and overall assay specificity.

3 | RESULTS

3.1 | Assay specificity

We developed three species-specific TaqMan® assays that amplified (i) a 128-bp fragment of the mtDNA DLoop region of *C. carcharias*, (ii) a 228-bp fragment of the mtDNA ND5 region of *C. leucas*, and (iii) a 92-bp fragment of the mtDNA DLoop region of *G. cuvier*. Each assay was shown to be highly specific to the target species when tested on DNA extracted from multiple tissue samples from 11 shark species captured in the area of this study (Table 2). This included six closely related species from the same genus as *C. leucas*. The multispecies

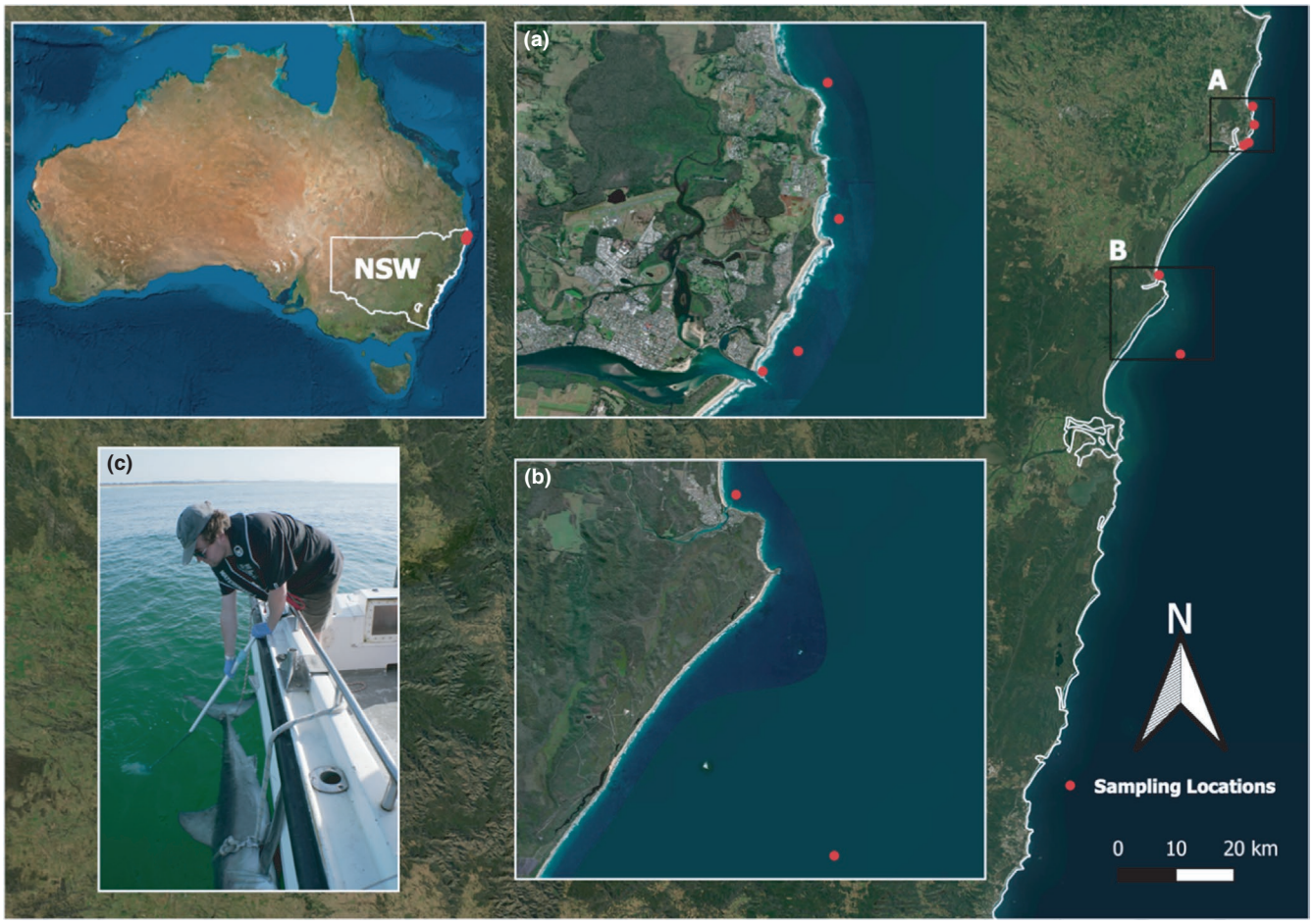

FIGURE 1 Location of water sampling sites (red circles) on the northern New South Wales (NSW) coast of eastern Australia. Insets (a) Ballina/Lennox Heads coastline, (b) Evans Head, and (c) eDNA sampling next to a *C. carcharias* capture

TABLE 2 Specificity of eDNA assays on target and non-target shark species found in the study region on the northern NSW coast of eastern Australia

Species	Common name	White shark (*cyt B*)[a]	Bull shark (ND2)[b]	White shark (DLoop)[c]	Bull shark (ND5)[c]	Tiger shark (DLoop)[c]
Carcharodon carcharias	White shark	✓ (29)	×	✓ (29)	×	×
Carcharhinus leucas	Bull shark	×	✓ (30)	×	✓ (30)	×
Galeocerdo cuvier	Tiger shark	×	×	×	×	✓ (29)
Isurus oxyrinchus	Mako shark	✓ (33)	×	×	×	×
Carcharhinus melanopterus	Blacktip reef shark	×	×	×	×	×
Sphyrna zygaena	Smooth hammerhead shark	×	×	×	×	×
Carcharhinus obscurus	Dusky shark	×	×	×	×	×
Carcharias taurus	Greynurse shark	×	×	×	×	×
Carcharhinus falciformis	Silky shark	×	✓ (31)	×	×	×
Carcharhinus brachyurus	Bronze whaler	×	✓ (29)	×	×	×
Carcharhinus brevipinna	Spinner shark	×	×	×	×	×

Note: Tissue samples for target and non-target shark species were normalized to 10 pg/µl. Values in brackets are the cycle quantification (Cq).

[a]Lafferty et al., 2018.

[b]Schweiss et al. (2020).

[c]This study.

TABLE 3 Water samples collected on the coast of northern New South Wales and screened with the multispecies assay for the presence of *G. cuvier* (T1–10), *C. carcharias* (W11–20), *C. leucas* (B21–30) and number of replicate qPCRs where a species is detected from a water sample (out of 3) is shown

ID	Latitude	Longitude	Sampling date	*C. carcharias*—FAM (pg)	*C. leucas*—Cy5 (pg)	*G. cuvier*—HEX (pg)
T1	−29.09817	153.43827	01/05/20	0	0	3 (1.6×10^4)
T2	−29.09817	153.43827	01/05/20	0	0	3 (4.9×10^3)
T3	−28.88078	153.58958	03/05/20	0	0	3 (3.8×10^2)
T4	−28.88078	153.58958	03/05/20	0	0	3 (4.1×10^2)
T5	−29.25602	153.47617	14/05/20	3 (3.5)	0	3 (8.8×10^3)
T6	−29.25602	153.47617	14/05/20	3 (0.2)	0	3 (6.3×10^2)
T7	−29.25208	153.47835	14/05/20	1	0	3 (1.2×10^3)
T8	−29.25208	153.47835	14/05/20	0	0	3 (17.5)
T9	−29.20652	153.48883	14/05/20	0	0	3 (5.6×10^4)
T10	−29.20652	153.48883	14/05/20	0	0	3 (3.2×10^3)
W11	−28.78881	153.60021	14/08/19	3 (10.1)	0	0
W12	−28.78881	153.60021	14/08/19	3 (12.7)	0	0
W13	−29.09022	153.44256	25/08/19	3 (6.7)	0	0
W14	−29.09022	153.44256	25/08/19	3 (53.3)	0	0
W15	−28.85970	153.60406	14/08/19	3 (1.1×10^3)	0	0
W16	−28.85970	153.60406	14/08/19	3 (1.7×10^2)	0	0
W17[a]	−28.79042	153.60049	14/08/19	3 (7.9)	0	0
W18[a]	−28.79042	153.60049	14/08/19	3 (12.4)	0	0
W19[b]	−29.09632	153.43836	13/08/19	3 (24.9)	0	0
W20[b]	−29.09632	153.43836	13/08/19	3 (7.3)	0	0
B21	−28.87499	153.59265	08/05/20	1	0	3 (2.3×10^4)
B22	−28.87499	153.59265	08/05/20	3 (4.0)	0	3 (0.8)
B23	−28.87499	153.59265	10/05/20	0	0	0
B24	−28.87499	153.59265	10/05/20	0	0	2
B25	−28.86931	153.60053	13/05/20	2	0	2
B26	−28.86931	153.60053	13/05/20	1	1	0
B27	−28.87499	153.59265	14/05/20	1	0	2
B28	−28.87499	153.59265	14/05/20	1	0	2
B29	−28.87499	153.59265	20/05/20	1	1	2
B30	−28.87499	153.59265	20/05/20	0	0	3 (2.2)
C31	−28.78881	153.60021	14/08/19	0	0	0
C32	−29.09022	153.44256	25/08/19	0	0	0
C33	−29.09022	153.44256	25/08/19	0	0	0
C34	−29.10885	153.43978	14/11/19	0	0	0
C35	−29.10885	153.43978	14/11/19	0	0	0
C36	−29.10885	153.43978	15/11/19	0	0	0
C37	−29.10885	153.43978	15/11/19	0	0	0
C38	−29.10885	153.43978	16/11/19	0	0	0
C39	−29.10885	153.43978	16/11/19	0	0	0
C40	−29.10885	153.43978	17/11/19	0	0	0
C41	−29.10885	153.43978	17/11/19	0	0	0
C42	−29.10885	153.43978	18/11/19	0	0	0
C43	−29.10885	153.43978	18/11/19	0	0	0

Note: Samples C31-33 are field sampling negative controls, and samples C34-43 are samples taken at Evans Head VR4G over five consecutive days. Numbers in brackets represent the amount of DNA (picograms) per liter, and only shown where all three replicates were positive.

[a]Collected ~250 m south of a *C. carcharias* capture.

[b]Collected 250 m north of a *C. carcharias* capture.

assay gave the same results even though we changed to PrimeTime assays and different fluorophores for *C. leucas* and *G. cuvier*.

We also tested the specificity of the *C. carcharias* assay from Lafferty et al. (2018) and the *C. leucas* assay from Schweiss et al. (2020) on the same 11 shark species found in our study area. These assays were found to amplify their respective target species, but also non-target species; the *C. carcharias* assay amplified the DNA of *I. oxyrinchus*, while the *C. leucas* assay amplified DNA from both *C. falciformis* and *C brachyurus* (Table 2).

3.2 | Assay efficiency and sensitivity

The standard curves produced from the serial dilution of each target species DNA showed a linear relationship between Cq value and the log of the starting DNA concentration (R^2 > 0.99 for all three assays). Each assay slope was −3.508, −3.452, and −3.326 corresponding to assay efficiencies of 93%, 95%, and 100% for *C. carcharias*, *C. leucas*, and *G. cuvier*, respectively. The LOD was 0.080 pg, 0.473 pg, and 0.582 pg and the LOQ was 0.596 pg, 1.037 pg, and 0.583 pg for *C. carcharias*, *C. leucas*, and *G. cuvier*, respectively.

3.3 | Field eDNA samples

The multispecies assay detected *G. cuvier* DNA strongly in 13 field samples (T1-10, B21-22, and B30) with all technical replicate qPCRs being positive (Table 3). Weaker detections were also found in another five samples, where only one or two of the technical replicate qPCRs were positive. The highest concentrations of DNA were found in samples taken from within 2 m of the *G. cuvier* captures (indicated by the high DNA concentrations in Table 3). *C. carcharias* DNA was strongly detected in 13 field samples (T5-6, W11-20, and B22) with all technical replicate qPCRs being positive (Table 3). This included four samples that were collected approximately 250 m from the site of capture and release of a *C. carcharias* individual approximately 15 min after its release. The species DNA was also detected in another seven samples, although only one or two out of three technical replicate qPCRs were positive in these samples. The multispecies assay detected *C. leucas* DNA weakly in only two samples (B26 and B29), both of which only one of three replicate qPCRs were positive. All detections were above the minimum LOD for each assay.

The three negative field control samples did not detect white shark DNA despite each being taken at the same time as white sharks were captured, indicating that our sampling method is robust to contamination. Similarly, all samples taken over five consecutive days at the Evans Head VR4G listening station (positioned 500 m offshore) did not detect white, tiger, or bull shark DNA, indicating no activity of these species, which is consistent with no captures or acoustic detections in the area for a period of 7 days prior and during the sampling period.

Sanger sequencing of qPCR amplicons confirmed species haplotype authenticity in seven *G. cuvier* DNA samples (T1-2, T4-5, B22, B25, B30) and six *C. carcharias* DNA samples (T5, W14-16, B22, and B25). Detections of both *G. cuvier* and *C. carcharias* DNA in the same samples were confirmed in three samples (T5, B22, and B25). We were unable to confirm product authenticity of *C. leucas* qPCR detections via Sanger sequencing due to low DNA concentrations in the two samples where the species was detected.

4 | DISCUSSION

The increasing frequency of human–shark interactions on the east coast of Australia has prompted investment into intensive shark monitoring programs in recent years (Colefax et al., 2020; Spaet et al., 2020b; Tate et al., 2019). In this study, we developed a highly specific multispecies eDNA assay that will provide added capabilities for detecting and discriminating between the three shark species responsible for the majority of human–shark interactions in eastern Australia (McPhee, 2014). The assay was shown to be effective at detecting the DNA of *C. carcharias*, *G. cuvier*, and to a lesser extent *C. leucas* from 1 L water samples collected off the coast of northern New South Wales, Australia. All water samples collected in close proximity (<2 m) to sharks captured and secured beside the research vessel (*C. carcharias* = 6 samples and *G. cuvier* = 10 samples) returned strong positive eDNA detections. Importantly, four water samples collected 250 m from the capture/release site for two *C. carcharias* also returned strong positive detections, indicating that spatial detectability of the eDNA assay persists over a wider area. No white shark DNA was detected in negative field control samples, indicating that our sampling approach was not causing false-positive detections. Further research is needed to understand the actual spatio-temporal limitations of the assay under different environmental conditions to help understand what a detection actually means in terms of real-time shark presence within the area. However, this is an important first step in the validation of the eDNA multispecies assay as a tool for detecting shark presence within an area and potentially gaining a greater appreciation for shark spatial ecology.

Our field surveys using the newly developed multispecies eDNA assay demonstrated the capability of detecting multiple species from a single water sample, with strong detections of both *C. carcharias* and *G. cuvier* DNA in multiple water samples collected in autumn 2020 (e.g., T5, T6, B22, B24). *Carcharodon carcharias* typically move northward along the east coast of Australia during autumn–winter months (Bruce et al., 2019; Spaet et al., 2020a), with near-shore visitation highest between July and December when annual sea-surface temperatures are at their lowest (Spaet et al., 2020a, 2020b). Conversely, *G. cuvier* tend to be found in warmer northern waters and typically move southward, with near-shore visitation peaking in summer and autumn, although they are found in NSW waters year-round (Holmes et al., 2014; Lipscombe et al., 2020). There is considerable overlap in near-shore visitation during autumn in Australia for both species, so it is not surprising that we were able to detect both species from water samples

collected at this time of year. This is an important finding given the risks both species pose to beachgoer safety at similar times of the year and suggests that eDNA approaches could be used to gain a better appreciation of spatial and temporal patterns of near-shore habitat use by each species.

The eDNA detections of *C. leucas* from two field samples were relatively weak, compared with some of the *C. carcharias* and *G. cuvier* detections. We did not capture any *C. leucas* during the sampling period and therefore could not directly sample water in an area with a confirmed presence. While present all year round within the study area, *C. leucas* are generally in lower densities and less active during daylight hours than either *C. carcharias* or *G. cuvier* and tend to occur in adjacent river systems (Werry et al., 2011). It is also worth noting that the *C. leucas* qPCR assay targets a larger amplicon (228 bp) compared to the *C. carcharias* (128 bp) and *G. cuvier* (92 bp) assays, which may affect field detectability due to the propensity for larger amplicons to degrade more quickly. Unfortunately, discrimination between *C. leucas* mtDNA sequences and non-target shark species was difficult and was only possible with a larger amplicon (the assay in Schweiss et al. (2020) is also of a similar amplicon size). Further testing is therefore recommended to assess whether amplicon size affects detectability of *C. leucas* from water samples. Ideally, this would be undertaken with known presence samples (e.g., water samples adjacent to *C. leucas* captures).

The multispecies assay developed here appears to be more specific than other eDNA-based assays developed for *C. carcharias* (Lafferty et al., 2018) and *C. leucas* (Schweiss et al., 2020), at least in our study region. Both of these previously developed assays detected non-target shark species occurring in eastern Australia, which compromises the use of these particular assays in this region. We expect our multispecies assay to be specific to *C. carcharias*, *C. leucas*, and *G. cuvier* populations in other regions of the world, as our primers and probes for each species were designed based on all available data on sharks in GenBank (Table S1). However, further specificity testing in regions where shark community assemblages are notably different is required to exclude the potential for cross-amplification of local non-target shark species.

The multispecies eDNA assay developed in this study will add to the shark detection capabilities of bather protection programs and provide an alternative tool for monitoring shark populations, that currently includes drumlines for capture (Tate et al., 2019), acoustic and satellite tagging and detection (Spaet et al., 2020a, 2020b), and drones (Butcher et al., 2019; Colefax et al., 2020), all paired with the SharkSmart app to provide real-time updates to the general public. Current automated acoustic monitoring techniques are limited to the detection of tagged individuals, which are likely to be only a small fraction of the total shark population present in eastern Australia (Davenport et al., 2021). Our eDNA assay has the potential to monitor a greater proportion of the shark population, particularly along coastal regions in hotspot areas at a fraction of the cost of current traditional monitoring approaches. Recent technological advances also allow in-field detection capabilities using mobile qPCR machines (Thomas et al., 2020), offering the potential for this multispecies eDNA assay to act as an early warning system to beachgoers for shark activity in the general area. Currently, the minimum time for extracting DNA and undertaking the multispecies assay is 6–24 hr from receipt of samples in a laboratory. An in-field detection system could cut this time down to 1–2 hr, making it possible for patrolled beaches to be tested routinely and results reported into shark management programs such as the SharkSmart app. Converting this assay for use with recombinase polymerase amplification (RPA) combined with a lateral flow strip detection method could further reduce time, costs and increase useability (Rohrman & Richards-Kortum, 2012). However, further research is needed to understand the spatial and temporal limitations of eDNA detections, particularly in the context of the real-time nature of a detection.

At present the primary driver for the development of innovative and cost-effective shark monitoring tools in Australia is around increasing public safety at beaches. However, strategic application of our multispecies eDNA assay has the potential to address critical knowledge gaps associated with the biology, ecology, and population dynamics of *C. carcharias*, *C. leucas*, and *G. cuvier* in Australia and potentially abroad. Other eDNA sampling and detection approaches (e.g., eDNA metabarcoding (Stat et al., 2017)) in combination with this multispecies assay could also be used to help understand the relationship between marine fauna and seasonal shark activity at hotspots. Similarly, targeted eDNA sampling across the species distribution (both in Australia and abroad) could greatly assist in improving our knowledge of the seasonality of shark movements and habitat use in both coastal and offshore environments.

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AUTHOR CONTRIBUTIONS

All authors were involved in the design of the study and contributed to the writing of the manuscript. ARW and ADM led manuscript preparation, AvR undertook assay design, specificity testing and eDNA sample analysis, and PAB and ZC undertook fieldwork.

DATA AVAILABILITY STATEMENT

All primers/probe sequences and results are available in the main text.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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