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RESOURCE ARTICLE

Detection and population genomics of sea turtle species via noninvasive environmental DNA analysis of nesting beach sand tracks and oceanic water

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Abstract

Elusive aquatic wildlife, such as endangered sea turtles, are difficult to monitor and conserve. As novel molecular and genetic technologies develop, it is possible to adapt and optimize them for wildlife conservation. One such technology is environmental (e)DNA – the detection of DNA shed from organisms into their surrounding environments. We developed species-specific green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) sea turtle probe-based qPCR assays, which can detect and quantify sea turtle eDNA in controlled (captive tank water and sand samples) and free ranging (oceanic water samples and nesting beach sand) settings. eDNA detection complemented traditional in-water sea turtle monitoring by enabling detection even when turtles were not visually observed. Furthermore, we report that high throughput shotgun sequencing of eDNA sand samples enabled sea turtle population genetic studies and pathogen monitoring, demonstrating that noninvasive eDNA techniques are viable and efficient

alternatives to biological sampling (e.g., biopsies and blood draws). Genetic information was obtained from sand many hours after nesting events, without having to observe or interact with the target individual. This greatly reduces the sampling stress experienced by nesting mothers and emerging hatchlings, and avoids sacrificing viable eggs for genetic analysis. The detection of pathogens from sand indicates significant potential for increased wildlife disease monitoring capacity and viral variant surveillance. Together, these results demonstrate the potential of eDNA approaches to ultimately help understand and conserve threatened species such as sea turtles.

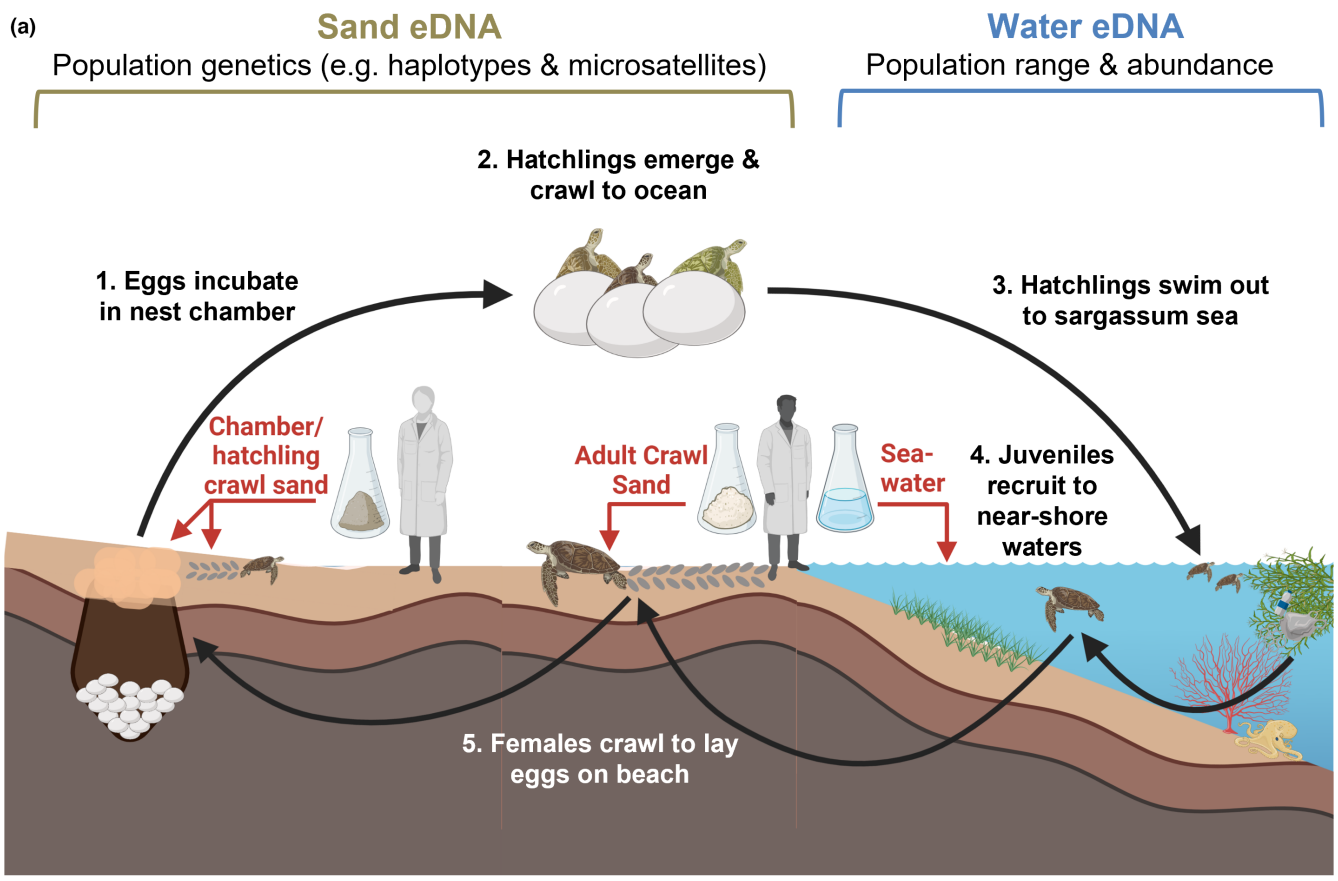
KEYWORDS
ChHV5, endangered species, environmental DNA (eDNA), pathogens, population genetics/genomics, population monitoring, sea turtles

1 | INTRODUCTION

Wild sea turtle populations are threatened as a result of natural and anthropogenic factors (Duffy et al., 2018; Eastman et al., 2020; Hamann et al., 2010; IUCN, 2020; Jones et al., 2016; Mashkour et al., 2020). Many of their natural disease threats have been exacerbated by human interaction in the last several decades (Jones et al., 2016; dos Santos et al., 2010; Whilde et al., 2017). It is exceedingly difficult to conserve these threatened wild populations due to the low-density and highly mobile nature of marine turtles. During ontogeny, each species' life-stage is characterized by habitat shifts, whether that is the rapid dispersal from near-shore to off-shore sargassum of post-hatchling green sea turtles (*Chelonia mydas*), congregation on nesting beaches and warm coastal waters of nesting adult loggerheads (*Caretta caretta*), or prolonged coastal habitation of juvenile greens (Avens et al., 2003; Eastman et al., 2020; Hardy et al., 2018; Ingels et al., 2020; Pfaller et al., 2020; Rodriguez & Heck, 2020). Little is known about the shifting dynamics of these populations, or their habitat adaptations in response to the rapidly changing ocean/coastal environment. Furthermore, detecting species abundances and ranges in general is more challenging in marine environments than terrestrial due to difficulty of access (Adams et al., 2019; Andruszkiewicz et al., 2017; Lacoursiere-Roussel et al., 2016). Traditional research methods have included capture/observation-based surveys that can be expensive, invasive and inefficient (Blasi & Mattei, 2017; Herren et al., 2018; Whiting et al., 2014). However, with the continuing development of novel molecular/genetic technologies, coupled with emerging environmental sampling approaches, the accuracy and efficiency of marine ecosystem monitoring and vulnerable species conservation is improving rapidly (Qu & Stewart, 2019; Raemy & Ursenbacher, 2018). Environmental DNA (eDNA) is such an approach, enabling the forensics detection of genetic material shed into the environment (e.g., from skin, hair, scales, saliva, faeces, urine and blood) (Beng & Corlett, 2020). Environmental DNA has been successfully retrieved from water, permafrost, snow, air, soil, and sand (Barnes & Turner, 2016; Barnes et al., 2014; Davy et al., 2015; Kelly et al., 2014; Rees et al., 2014). These technological approaches will be a crucial future conservation tool for detecting and monitoring low-density threatened turtle species (Beng & Corlett, 2020; Farrell et al., 2021 et al., 2021; Farrell, Yetsko, et al., 2021; Harper et al., 2020; Jerde et al., 2011; Veilleux et al., 2021; Yetsko et al., 2021).

Efficient, accurate methods for tracking and analysing dynamic sea turtle populations, and noninvasive approaches for evaluating the population genetics among various nesting aggregations, could provide unique insight into these complex life-history stages. Environmental approaches enable the recovery of informative genetic material from study species without requiring difficult in-water siting and capture or having to be physically present during nesting events. Environmental DNA-based detection of sea turtle species in marine environments has been limited to just a few studies in limited circumstances (Farrell, Harper et al., 2020; Kelly et al., 2014; Shamblin et al., 2011; Yetsko et al., 2020, 2021), and detection and "genetic fingerprinting" of sea turtle species from beach sand traversed by nesting females and hatchlings has not yet been explored (Figure 1a).

While sea turtle species' ranges and distributions have altered over the years, so too have those of their pathogens, including fibropapillomatosis (FP), now afflicting every sea turtle species in every ocean globally (Eastman et al., 2020; Farrell et al., 2018; Farrell, Yetsko, et al., 2021; Jones et al., 2016; Smith & Coates, 1938; Williams et al., 1994; Yetsko et al., 2021). We recently employed eDNA-based approaches to study the shedding of the virus (ChHV5) associated with this disease, and these results, combined with the results of this study, can provide insight into the health and population status of wild sea turtles inhabiting our study sites (Farrell, Yetsko, et al., 2021; Yetsko et al., 2020). With increasing habitat degradation and vulnerability to threats, already threatened sea turtle populations are at increasing risk, therefore pathogen and population monitoring is crucial to aiding their survival (Crowe et al., 2020; Eastman et al., 2020; Gomez-Ramirez et al., 2020; Marn et al., 2017). In this study we developed and validated qPCR assays in a sea turtle rehabilitation setting and we conducted field trials to detect,



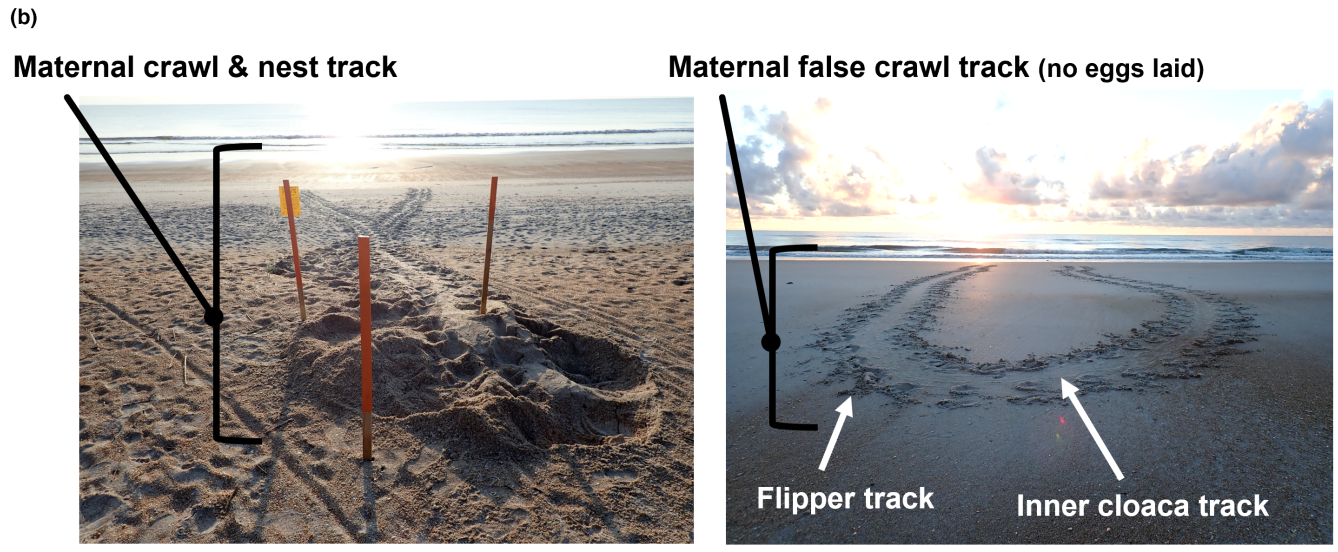


FIGURE 1 (a) Schematic of the potential noninvasive applicability of eDNA sand or water sampling to all sea turtle life stages. (b) Maternal nesting crawl track and nest the morning after laying (left). Maternal false crawl track with outer flipper and inner cloaca tracks (right). Photographs from Micklers Beach Sea Turtle Nesting Patrol, taken by Nancy Condron

quantify and assess sea turtle species (*C. mydas*, *C. caretta*) and their pathogens (ChHV5) in environmental seawater and sand settings. The results of this study provide support for the use of qPCR, PCR and sequencing-based eDNA analysis to detect, monitor, genetically fingerprint/trace and ultimately help understand and conserve threatened sea turtle species.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Sand (nine rehabilitation and 25 field, not including biological replicates) and water samples (48 rehabilitation and 23 field, not

including biological replicates) were collected (2017 to 2021) from rehabilitation settings and wild sites (Figures S1a,b), as detailed below. Rehabilitation samples were collected from seawater tanks with one-two juveniles or up to 32 post-hatchling washbacks, and sand enclosures with one-three hatchlings. Wild samples were collected from nine oceanic sites and four sand beaches (three nests and seven maternal crawls). Post-hatchling washbacks are defined as sea turtles that have emerged from the nesting beaches and have begun to feed in the ocean, but who have been washed back ashore (i.e., stranded), often due to storm activity (Eastman et al., 2020). Sand samples were collected by scraping along the upper surface of the sand track (where genetic material was most likely to be shed) using a sterile 50 ml Falcon tube (Fisher Scientific and Corning). For post-emergence nest sand samples, a Falcon tube was filled from the nest sand spoil heap after nest evaluation activities had been completed. For individual hatchling crawls, the Falcon tube was sufficiently wide to cover the width of the entire crawl. For large nesting female crawl tracks separate sand scrapes were taken from the inner cloaca part of the crawl and the outer flipper area of the crawl. Nesting female crawls are horseshoe-shaped, starting and finishing where the sand meets the ocean, therefore samples were collected as close to the apex of the horseshoe (nearest the potential nest without nest interference, and furthest away from the water where genetic material could be washed away) as possible. All wild maternal sand samples were collected after the mothers had returned to the ocean, either on the day the tracks were created (less than 12 h after the crawl) or the following day (over 24 h after the crawl). False crawls/nests (where a female crawls up the beach and begins to dig a nest, but abandons it without laying eggs) and successful nesting crawls/nests were all sampled (Figure 1b) – the duration of time and level of activity at both the false sites and the successful sites are relatively similar; however, it may be assumed that less cloacal fluid would potentially be shed (a source of eDNA) at the false sites where egg laying has not occurred. Individual hatchling tracks were sampled shortly (minutes) after they were made by the hatchling. Sampling equipment was disinfected with 10% bleach and rinsed thoroughly with deionized water prior to use (water collection) and disposable nitrile gloves were worn. Sand samples (50 ml each) were collected by hand using sterile 50 ml Falcon conical centrifuge tubes (Fisher Scientific and Corning). Water samples (500 ml–1 L each) were collected by hand using sterile 500 ml Nalgene HDPE plastic bottles (Fisher Scientific). After collection, sand and water samples were immediately placed in a dark sterile cool box and transported to the University of Florida's Whitney Laboratory for Marine Bioscience, St Augustine Florida. All water samples were filtered on the collection day (within 8 h). All sand samples were filtered on the same day, except for False Crawl 3, 4, 5 and 6 samples that were frozen at −20°C for 3 weeks prior to extraction (Table S1). In addition, three wild sand samples were stored at −20°C for three months to assess the effectiveness of prolonged freezing for preserving sand samples (Table S2). For negative field control sand sampling, 50 ml dry sand was collected away from suspected turtle presence (away from turtle tracks) on each sampling trip. For negative field control water sampling, 1 L MilliQ water was transported from the laboratory to rehabilitation or wild sampling locations and stored in a cool box with the environmental samples to monitor for potential contamination during sampling and transportation. Water and sand negative field controls were filtered and extracted alongside the other collected sand/water samples from each sampling trip and subjected to the same qPCR or next generation sequencing conditions.

Marine leeches were also removed from juvenile sea turtle patients, as part of their standard rehabilitation care, and DNA was extracted (DNeasy blood and tissue kit, Qiagen) to determine whether sea turtle species-specific detection was possible from leech blood pellets, as a potential alternative field method for detecting sea turtle presence in the absence of visual sightings. For detailed sampling and DNA extraction methods please see the Supporting Information Methods (and Tables S1–S10).

2.2 | DNA amplification: qPCR

The qPCR reaction mixtures were performed in a total volume of 10 µl: 5 µl TaqMan Fast Advanced Master Mix (Fisher Scientific cat no: 4444557); 3 µl Nuclease free water (Fisher Scientific); 1 µl ChHV5 DPol. (Mashkour et al., 2021) or custom *C. mydas 16S rRNA* or custom *C. caretta 16S rRNA* (Table S3) 5 µM primer/probe stock (final concentration of 0.5 µM of each primer/probe); 1 µl DNA template, per well of a 384-well plate.

Additional qPCR reactions were run to test for pan-eukaryotic eDNA presence (Applied Biosystems Pan-eukaryotic *18S rRNA* assay, cat no: 4352930E). This acted as a positive control for the eDNA extraction. Pan-eukaryotic *18S rRNA* qPCR reaction mixtures were also performed in a total volume of 10 µl: 5 µl TaqMan Fast Advanced Master Mix; 3.5 µl Nuclease free water; 0.5 µl *18S rRNA* pan-eukaryotic assay (manufacturer supplied 20x concentration); 1 µl DNA template.

Depending on the sample type, stage of methodology optimization and the sample volume available, each sample was run in 3–24 technical replicates (Tables S1, 2, 4–8). In general, we selected three technical replicates for rehabilitation tank water, six technical replicates for rehabilitation and beach sand samples and 24 technical replicates for oceanic water samples. Three no template controls (NTCs) per assay were included as well as an assay-specific standard curve (ChHV5, *C. mydas* or *C. caretta*) consisting of six gene dilutions from 10 pg to 0.0001 pg (for synthetic gene fragment length and sequence of each sea turtle fragment see Table S3, for ChHV5 DNA polymerase [Dpol / UL30] gene fragment see Farrell, Yetsko, et al., 2021). qPCR reactions were performed on a Roche LightCycler 480 II or an Applied Biosystems QuantStudio 6 Pro, with Taqman Fast Advanced Master mix (Fisher Scientific cat no: 4444557).

Thermal cycling conditions were identical for each qPCR machine and were as follows: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15 s and 60°C for 1 min.

There are currently no set criteria for the minimum number of eDNA qPCR technical replicates required to amplify to confirm a positive result, or in this case species/pathogen-specific sea turtle/ChHV5 presence (Goldberg et al., 2016). Therefore, positive amplification of one or more technical replicates per sample was counted as positive detection. Amplification ratio (the proportion of positive amplification detection relative to attempted technical replicate reactions [Farrell, Yetsko, et al., 2021; Shamblin et al., 2011]) is reported for all study samples (Tables S1, 2, 4–8).

For qPCR assay design and tissue haplotyping by conventional PCR, please see the Supporting Information Methods.

2.3 | DNA sequencing

Nontargeted shotgun-based next generation sequencing was performed on select water and sand samples using either an Illumina HiSeq 3000 (paired-end 100 bp reads) or NovaSeq 6000 (paired-end 150 bp reads), at the University of Florida's Interdisciplinary Center for Biotechnology Research Core Facilities (Table S9). 1,065 million reads were generated from 11 water eDNA samples, and 2,404 million reads were generated from 10 sand eDNA samples. Samples we sequenced as described in Farrell, Yetsko, et al. (2021), for more details see the Supporting Information Methods. One pooled library of water eDNA samples, combining five rehabilitation tank water samples (Figure S2) from UF's Whitney Laboratory Sea Turtle Hospital was sequenced, four of these tanks housed juvenile *C. mydas* with one tank housing post-hatchling *C. caretta* (Farrell, Yetsko, et al., 2021). For this pooled sample, water collection, filtration and extraction were performed separately on each tank sample, and the final purified DNA was pooled prior to library preparation. Reads from this sample relating to the sea turtle viral pathogen (ChHV5) have recently been published (Farrell, Yetsko, et al., 2021). Pooling was conducted to obtain the average ChHV5 load across the five tanks. Except for this tank sample, no other water or sand samples were pooled prior to sequencing, with sequencing being performed on each individually extracted sample. A water sample from a tidal pool housing 26 *C. mydas* and 2 *C. caretta* at the Turtle Hospital, Marathon was also used. All sequenced samples including raw reads are deposited in NCBI (https://www.ncbi.nlm.nih.gov/) under BioProject ID: PRJNA449022 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA449022).

Using the Galaxy (https://usegalaxy.eu/) bioinformatics platform, raw reads underwent quality control checks with FastQC (Galaxy Version 0.72+galaxy1) and were then trimmed (Trim Galore! Version 0.4.3.1) and aligned to a reference *C. mydas* genome: GenBank accession number: GCA_000344595.1 used for water samples, and GenBank accession number: GCA_015237465.1 used for sand samples (Bentley et al., 2022; Wang et al., 2013). The newer *C. mydas* genome (GCA_015237465.1) had not been published at the time of the water sample analysis (Bentley et al., 2022). As there is no current reference *C. caretta* genome available, the *C. mydas* reference genomes were utilized for whole genome analysis for both species. Alignments were conducted using Bowtie2 (Galaxy Version 2.4.2+galaxy0) and HISAT2 (Galaxy Version 2.2.1+galaxy0). There is currently no *C. caretta* reference genome available for bioinformatic analysis, hence only the *C. mydas* reference genome was used for all whole genome sequencing analysis.

Reads obtained from the sand samples were also aligned to mitochondrial reference genomes for phylogenetic and haplotype analysis (independently of the genome alignments), as population genetics has been conducted on whole mitochondria and mitochondrial fragments for both species (Duchene et al., 2012; Komoroske et al., 2017; Shamblin, Bjorndal, et al., 2012; Shamblin et al., 2014, 2015; Shamblin, Bolten, et al., 2012). As sea turtle DNA either comes from a sand track from one individual, or a nest sample where all individuals share the same mother (inherited mitochondrial phylogeny), mitochondrial DNA can be used for accurate haplotyping. In comparison, water samples probably contain DNA from a mix of individuals, so haplotyping was not attempted for those samples. For mitochondrial genome analysis, trimmed sand reads were aligned to either a *C. mydas* or *C. caretta* [respective GenBank accession numbers: JQ034420.1, JX454983.1] whole mitochondrion reference genome using Bowtie2 (Duchene et al., 2012; Shamblin, Bjorndal, et al., 2012). Trimmed reads for all sand samples were also aligned (as above) to the ChHV5 reference genome (GenBank accession number: HQ878327.2, Ackermann et al., 2012).

All alignments were BAM filtered (Galaxy Version 0.5.9), keeping only the mapped reads. The mitochondrion alignments (BAM files) were used as input for Ococo (Galaxy Version 0.1.2.6) to generate consensus sequences for each sample. The reference mitochondrion genomes were selected as the "backbones" of the new consensus sequences. The strategy for building the consensus was performed on a majority basis, with Ococo inferring single nucleotide polymorphisms on a majority basis, and then constructing a new consensus sequence for downstream analysis. As a limited number of whole sea turtle mitochondrial sequences have been deposited on NCBI, we also compared sand eDNA-derived consensus sequences to a partial mitochondrial fragment (815 bp fragment for loggerhead and 817 bp for green turtles) for which a greater number of deposited sequences with more fine-grained geographic coverage exist. This region has also been used to define *C. caretta* haplogroups and haplotypes (LaCasella et al., 2007; Nielsen et al., 2011; Shamblin et al., 2014; Shamblin, Bolten, et al., 2012) and *C. mydas* lineages (E. Naro-Maciel, unpublished data; Shamblin et al., 2015; Shamblin, Bjorndal, et al., 2012). For phylogenetic tree construction, whole mitochondrial consensus sequences and the reference whole mitochondrial genome sequences retrieved from NCBI (Table S10) (for comparison) were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) to examine degrees of relatedness and to generate phylogenetic trees, using the neighbour-joining method.

ChHV5 consensus genome generation from sand eDNA and phylogenetic analysis was conducted as previously described in Whitmore et al. (2021), for the sample with sufficient ChHV5 aligning reads (patient "Archie").

3 | RESULTS

3.1 | Species-specific detection of loggerhead and green sea turtle eDNA from tank water samples - validation in a rehabilitation setting

Initial validation of the *16S rRNA* gene species-specific qPCR assays (Table S3), was conducted using eDNA extracted from five sea turtle rehabilitation tank water samples. Green (*C. mydas)* and loggerhead (*C. caretta)* sea turtle *16S rRNA* gene probe-based qPCR assays were developed for the detection of sea turtles from eDNA samples (Table S3). These assays were species-specific, with no cross-reactivity from available tissue, water eDNA (rehabilitation tank) or sand eDNA (beach sand) samples from five sea turtle species (*C. mydas*, *C. caretta*, *D. coriacea*, *L. kempii*, *L. olivacea*) (Figures S2, 3a, b). The *C. mydas* assay only detected *C. mydas* DNA, while the *C. caretta* assay only detected *C. caretta* DNA, with no cross-reactivity between species (Figures S2, 3a, b). These assays could also specifically detect whether marine leeches had been feeding on *C. mydas* or *C. caretta* blood (Figure S3c). The *C. mydas* assay could detect sequences matching both the Pacific and Atlantic *C. mydas* subpopulations (despite a one base pair mismatch in the Pacific population, Table S3) with high sensitivity (Figure S4).

All negative controls (water, sand, rehabilitation, wild) tested negative for all species- and pathogen-specific qPCR assays. This supports our equipment sterilization protocols and sterile sample collection techniques, and confirms that no contamination happened throughout the laboratory process, as contamination was avoided in all field (and no template qPCR) negative control biological and technical replicates. *C. mydas* eDNA was successfully amplified from all post-hatchling washback (newly hatched sea turtles who have made it from the nest to the ocean, but get washed back onshore by strong currents and transported to rehabilitation facilities to monitor before returning to the sea) and juvenile (Figure 2) rehabilitation water samples (amplification ratios from 0.667–1), with 99% of samples having an amplification ratio of 1 (i.e., amplification in all technical replicates) (Figure 2a; Table S6). There was a positive relationship between the abundance of *C. mydas* (washbacks) in the tank and the concentration of *C. mydas* eDNA detected (Figure 2a). As *C. mydas* abundance increased, so too did the concentration of *C. mydas* eDNA, providing evidence for the potential future use of this technique to track population abundances over time in a fixed location. *C. caretta* eDNA was successfully amplified from all washback rehabilitation water samples, bar one biological replicate, (amplification ratios from 0 – 1), with 83% of samples having an amplification ratio of 1 (Figure 2b; Table S7). There was a statistically significant positive correlation between the abundance of *C. caretta* washbacks and the concentration of *C. caretta* eDNA detected in the tank environment (Pearson correlation coefficient $R = 0.946$, $R^2 = 0.894$, $p = .004$, df = 5) (Figure 2b). As *C. caretta* abundance increased, so too did the concentration of *C. caretta* eDNA in the water.

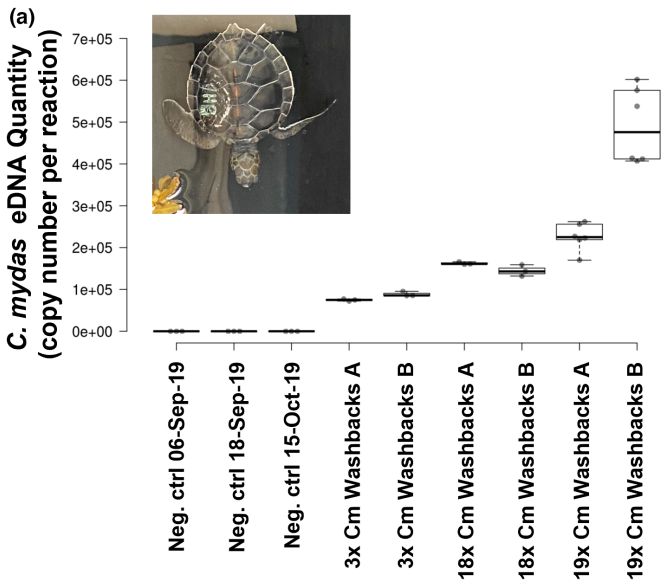
3.2 | Detection and quantification of sea turtle DNA from free-ranging turtles - oceanic eDNA field trial

Having confirmed the ability of this methodology to detect sea turtle eDNA in line with animal abundance in an aquatic rehabilitation setting, we next assessed the performance of the assays to detect and quantify eDNA from free-ranging turtles from water samples collected from the wild. *C. mydas* eDNA was successfully amplified from seven out of the nine different wild water macro-locations. representing 23 subsites (Figure S1; Table S8) (17 out of the 23 individual sites [Figure 2c]) throughout Florida (amplification ratios from 0–1). *C. mydas* could be detected by eDNA approaches in known green turtle habitats even when traditional observation-based approaches did not detect any *C. mydas* at the time (Figure 2c).

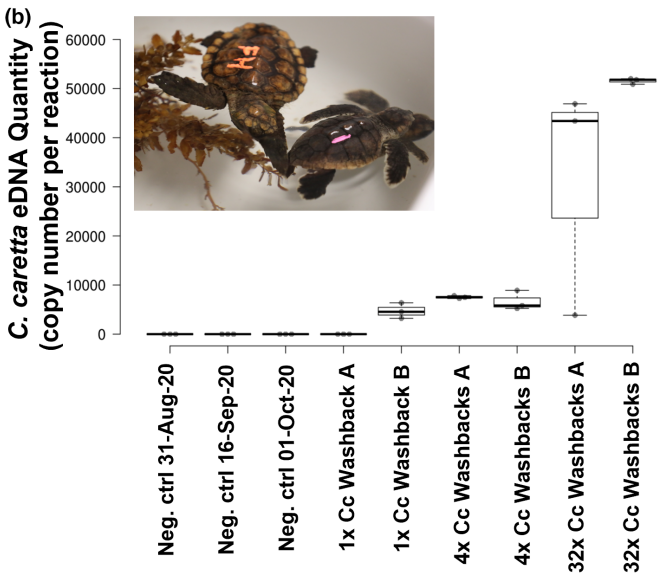
3.3 | Species-specific detection of *C. mydas* and *C. caretta* from sand eDNA - validation in a rehabilitation setting

Having confirmed the specificity of the new assays in aquatic tank water and ocean samples, we next assessed their suitability for the

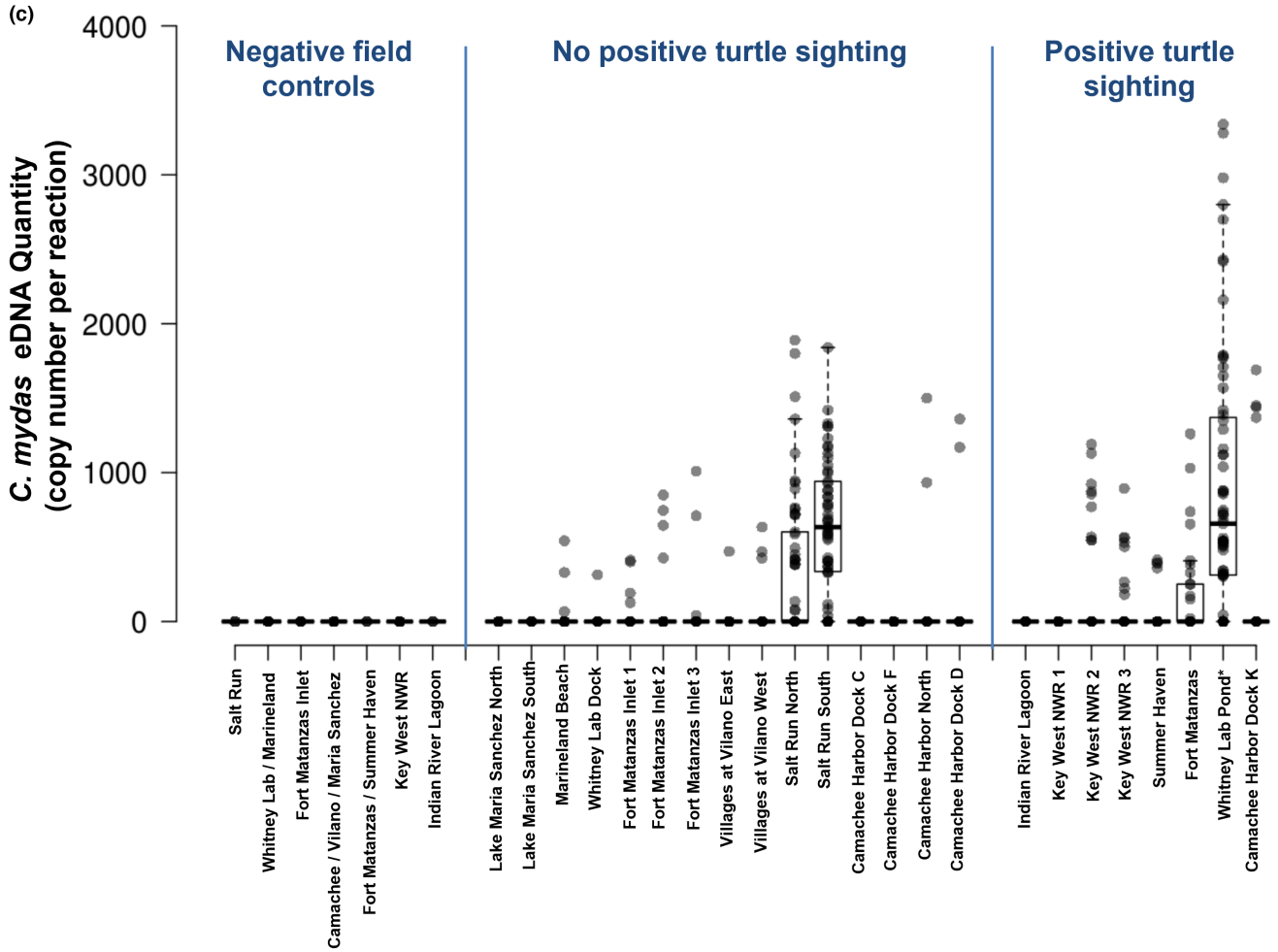
FIGURE 2 qPCR based species-specific quantification of sea turtle eDNA from rehabilitation tank and oceanic field water samples. (a) Quantity (copy number / reaction) of washback (post-hatchling) green turtle (*C. mydas*) eDNA in rehabilitation tank water samples. Number of washbacks indicated by label numbers. a–b refer to biological replicates. Each qPCR reaction is a 10 μl reaction containing 1 μl of extracted eDNA template. Additional data can be found in Table S6. Inset image, a post-hatchling washback *C. mydas* in a rehabilitation water environment at the University of Florida's Whitney Sea Turtle Hospital. (b) Quantity (copy number / reaction) of post-hatchling washback loggerhead (*C. caretta*) eDNA in rehabilitation water samples. Number of washbacks indicated by label numbers. A–B refer to biological replicates. Each qPCR reaction is a 10 μl reaction containing 1 μl of extracted eDNA template. There was a positive correlation between the abundance of *C. caretta* and the quantity of *C. caretta* eDNA detected in the tank water samples (Pearson correlation coefficient $R = 0.946$, ($R^2 = 0.894$), $p = .004$, df = 5). Additional data can be found in Table S7. Inset image, *C. caretta* washbacks at the Whitney Sea Turtle Hospital. (c) Quantity (copy number/ reaction) of green turtle (*C. mydas*) eDNA in wild water samples, both when turtles were visible at the time of sample collection and when they were not. Samples were collected from several coastal and intracoastal sites along the east coast of Florida, from as north as Vilano to as south as Key West. The Whitney Laboratory Pond sample marked by an asterisk (*) is a hybrid wild/rehabilitation sample as it contains outflow from the Whitney Sea Turtle Hospital tanks. Each qPCR reaction is a 10 μl reaction containing 1 μl of extracted eDNA template. Additional data can be found in Figure S1 and Table S8



C. mydas post-hatchling washback eDNA quantity in rehabilitation tank water



C. caretta post-hatchling washback eDNA quantity in rehabilitation tank water



C. mydas eDNA quantity in wild oceanic water

detection of sea turtle DNA from sand samples, in a rehabilitation setting. In addition to the predominantly aquatic life-history of sea turtles, crucial maternal nesting and hatchling nest emergence events occur on beaches. Therefore, noninvasive sea turtle eDNA recovery from sand samples has potential conservation-relevant applications (Huerlimann et al., 2020). *C. mydas* eDNA was successfully amplified from all *C. mydas* hatchling and juvenile rehabilitation sand samples (amplification ratios from 0.5–1) with 94% of samples having an amplification ratio of 1 (Figure 3a,b; Table S4). *C. caretta* eDNA was successfully amplified from all *C. caretta* hatchling rehabilitation sand samples (amplification ratios from 0.333–1) with 50% of samples having an amplification ratio of 1 (Figure 3b; Table S5). This is the first reported detection of sea turtle eDNA from sand samples. More species-specific eDNA was detected in the *C. caretta* sand samples than the *C. mydas* sand samples, potentially due to the much higher activity rate of the *C. caretta* hatchlings and consequently the higher shedding of their genetic material into their environment (this eDNA shedding to activity/sand interaction trend was also observed with wild hatchlings, see below).

3.4 | Recovery of nesting female and emerged hatchling sea turtle DNA from beach sand –nesting beach sand wild field trial

Sea turtle DNA was readily retrievable from beach sand samples (maternal crawls, hatchling crawls and nest evaluation spoil heaps encompassing "Egg Chambers", "Nest Surfaces" and "Emergence Holes") (Figures 1b, 4, 5, Figures S5a–c), with no amplification from negative field controls (sand collected away from obvious

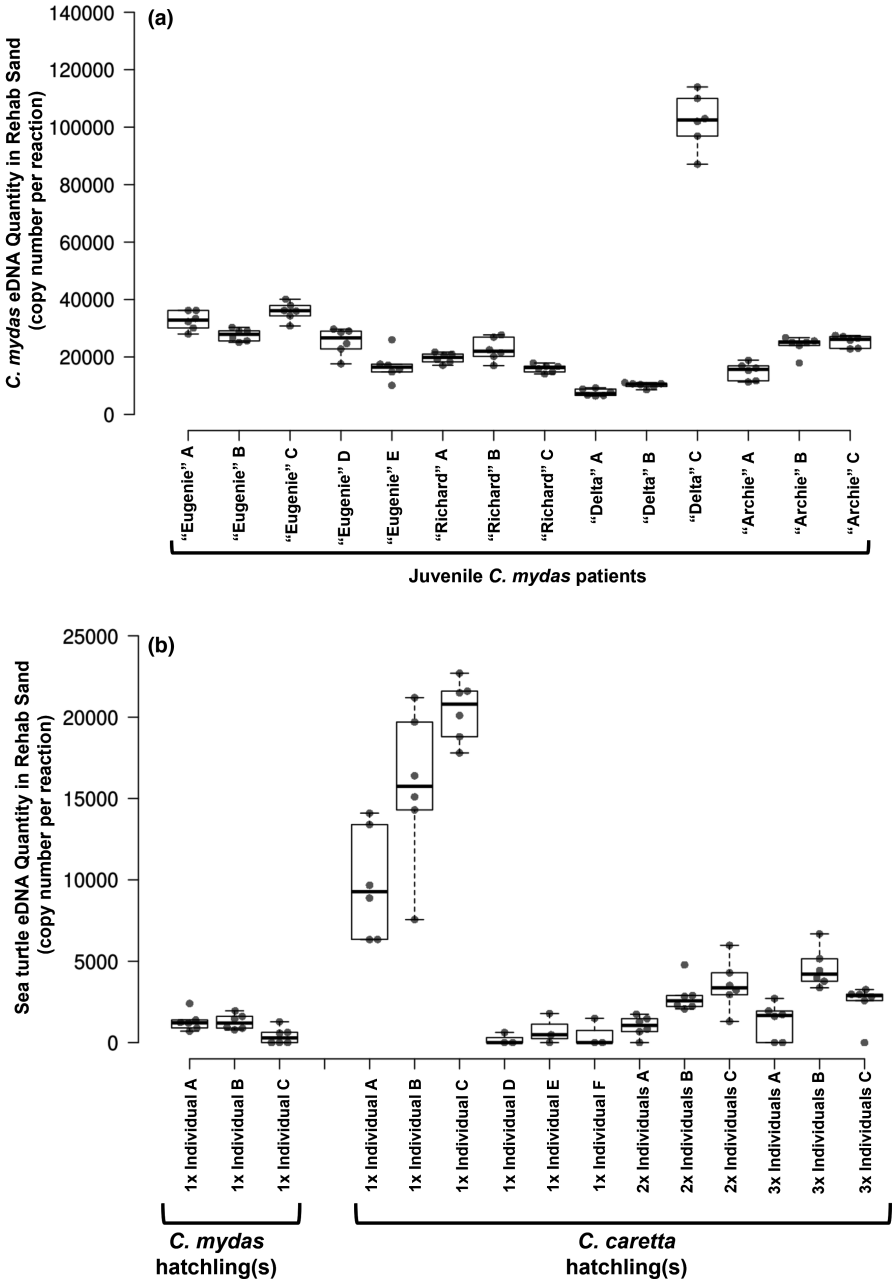


FIGURE 3 qPCR based species-specific quantification of sea turtle eDNA from rehabilitation sand samples. (a) Quantity (copy number / reaction) of juvenile green turtle (*C. mydas*) eDNA in rehabilitation sand samples. Each qPCR reaction is a 10 µl reaction containing 1 µl of extracted eDNA template. A–E refer to biological replicates. All samples were collected from the Whitney Sea Turtle Hospital. Additional data can be found in Table S4b) Quantity (copy number / reaction) of hatchling green turtle (*C. mydas*) and loggerhead (*C. caretta*) eDNA in rehabilitation sand samples. Each qPCR reaction is a 10 µl reaction containing 1 µl of extracted eDNA template. Number of hatchlings indicated by label numbers. A–C refer to biological replicates. All samples were collected from the Whitney Sea Turtle Hospital. Additional data can be found in Tables S4 and S5

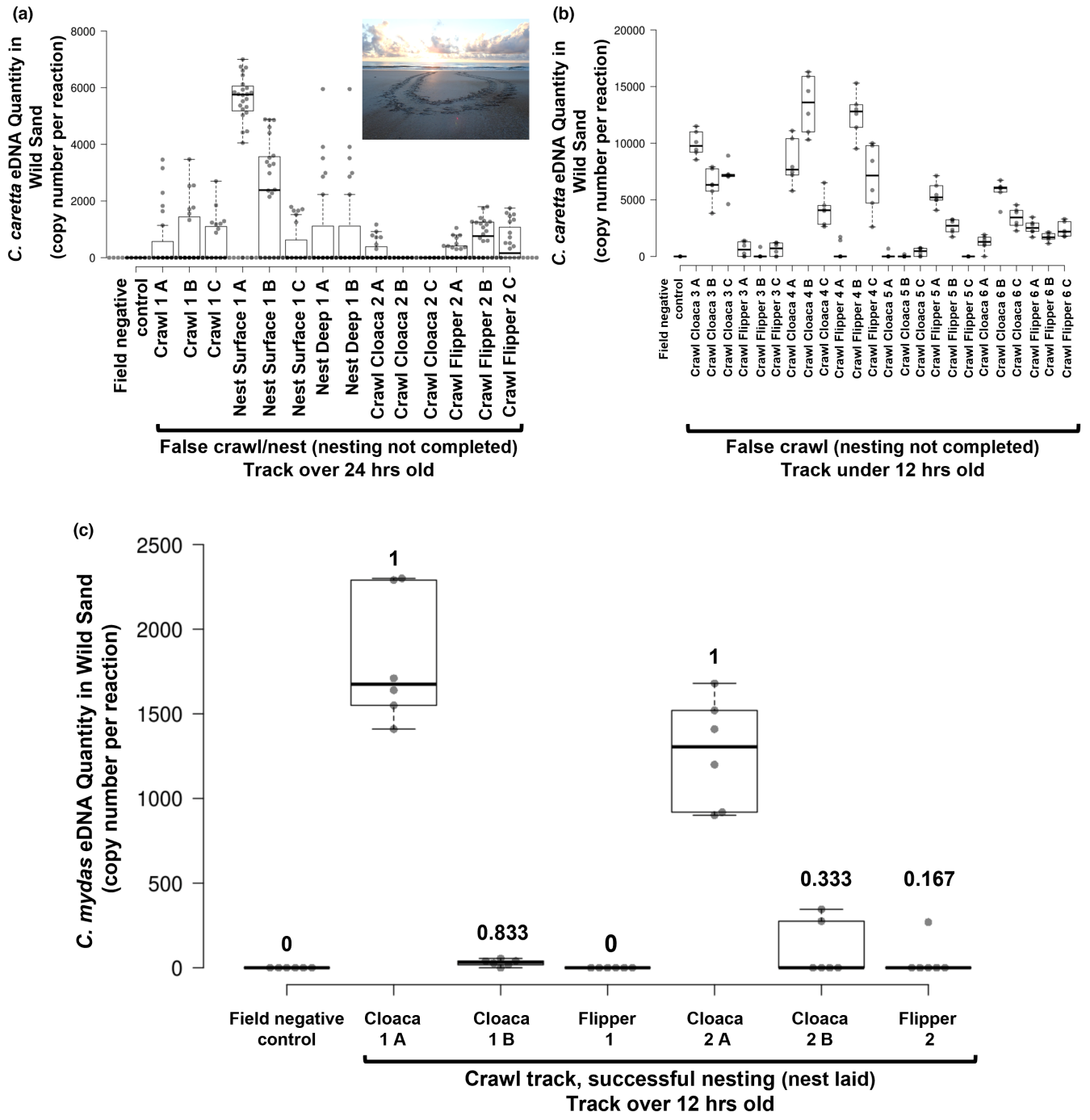


FIGURE 4 qPCR based species-specific quantification of sea turtle eDNA from sand field samples of crawl tracks from nesting females. (a) Quantity (copy number / reaction) of maternal loggerhead (*C. caretta*) eDNA in wild sand samples collected from "false" (non-nesting/aborted nesting) crawls collected over 24 hours post-crawl. Each qPCR reaction is a 10 µl reaction containing 1 µl of extracted eDNA template. Numbers refer to sample ID numbers, not number of turtles. A–C refer to biological replicates. All samples were collected from Guana Beach, FL. Flipper denotes sand was collected from the outer portion of the crawl disturbed by flippers, and cloaca denotes sand collected from the inside portion of the track. Additional data can be found in Table S1. Insert image, *C. caretta* non-nesting/aborted nesting "false" crawl. (b) Quantity (copy number / reaction) of maternal loggerhead (*C. caretta*) eDNA in wild sand samples collected from false crawls less than 12 h post-crawl. Each qPCR reaction is a 10 µl reaction containing 1 µl of extracted eDNA template. Numbers refer to sample ID numbers, not number of turtles. A–C refer to biological replicates. Samples were collected from Micklers, Marineland and Crescent Beaches, FL. Additional data can be found in Table S1. (c) Quantity (copy number / reaction) of maternal green (*C. mydas*) sea turtle eDNA in wild sand samples collected from nesting crawls less than 12 h post-crawl. Each qPCR reaction is a 10 µl reaction containing 1 µl of extracted eDNA template. Numbers refer to sample ID numbers, not number of turtles. A–B refer to biological replicates. Numbers above each column denote the amplification ratio for each sample (number of amplifying technical replicates divided by the number of technical replicates ran). Samples were collected from Marineland and Micklers Beaches, FL

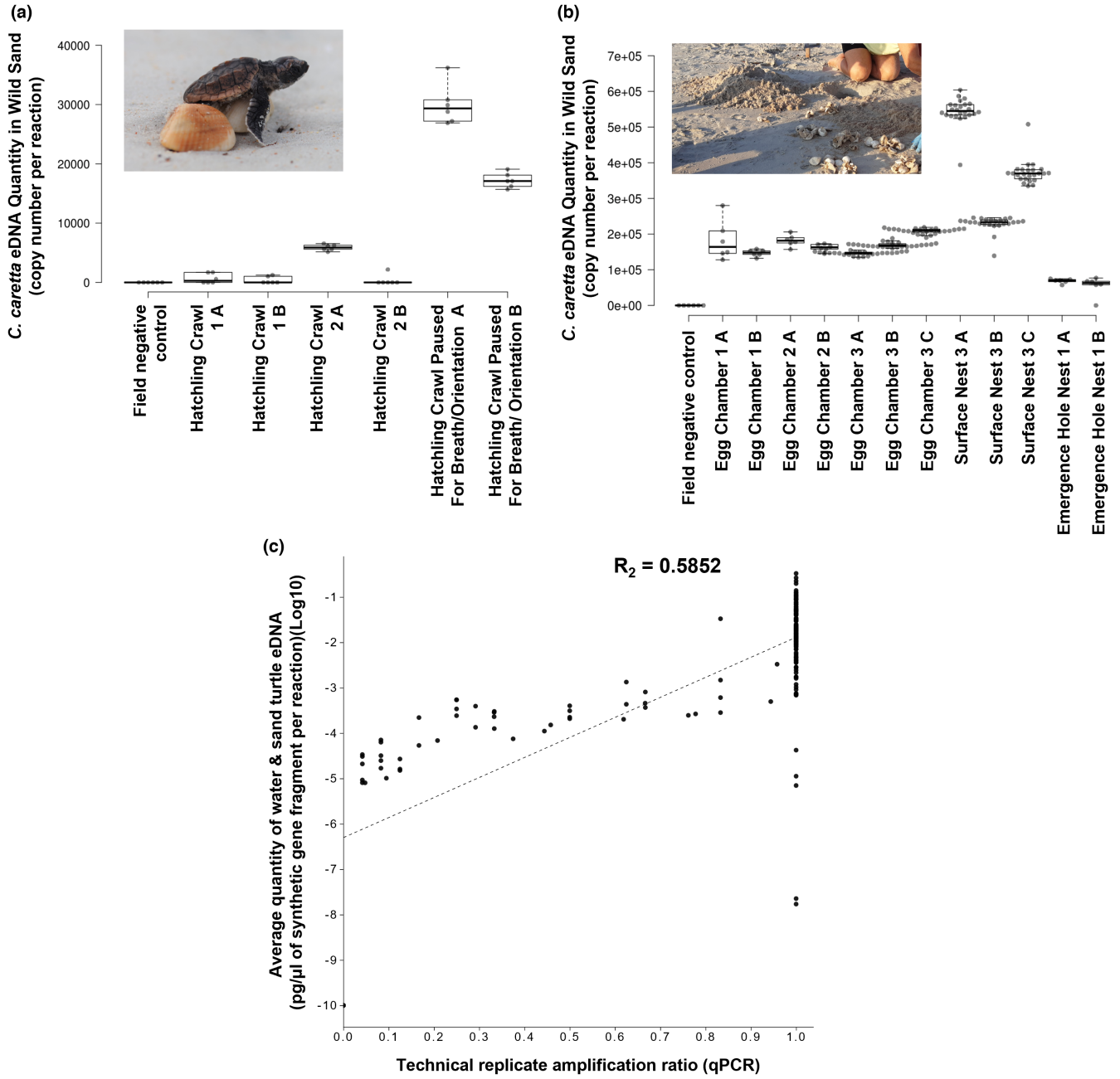


FIGURE 5 qPCR based species-specific quantification of sea turtle eDNA from sand field samples of hatchling tracks made after emerging from nests. (a) Quantity (copy number / reaction) of hatchling loggerhead (*C. caretta*) eDNA in wild sand samples collected from single hatchling crawls. Each qPCR reaction is a 10 µl reaction containing 1 µl of extracted eDNA template. Numbers refer to sample ID numbers, not number of turtles. A–B refer to biological replicates. All samples were collected from the same nest emergence on Marineland Beach, FL. Samples were collected from "active" continuous forward crawl portions of the track, or from tracks where the same hatchling stopped its forward momentum and moved in circles (probably orientating) on an isolated spot and took a breath (involves downward motion of front flippers). Additional data can be found in Table S1. Inset image, a *C. caretta* hatchling crawling from its nest towards the ocean. (b) Quantity (copy number / reaction) of hatchling loggerhead (*C. caretta*) eDNA in wild samples collected from multiple groups of hatchlings emerging from nests (nest spoil heaps). Numbers refer to sample ID numbers, not number of turtles. A-C refer to biological replicates. Samples were collected from Marineland and Guana Beaches, FL. Additional data can be found in Table S1. Inset image, a post *C. caretta* nest evaluation sand spoil heap. (c) Strong positive correlation between the quantity (pg/µl of sea turtle 16s rRNA gene eDNA from 1 µl DNA template in a 10 µl qPCR reaction, based on the standard curve of a known quantity of 16s rRNA synthetic gene fragment) of sea turtle eDNA in water and sand, and the amplification ratio of their technical replicates (Pearson correlation coefficient: $R = 0.765$, $R^2 = 0.5852$, $p < .00001$). Data was log10 transformed and all values of 0 were converted to −10

sea turtle activity [Figures 4a–c, 5a, b]). *C. caretta* eDNA was successfully amplified from all sampled wild beach sand locations in northeast Florida (biological replicate amplification ratios from 0 [6%] – 1 [44%]) (Figures 4, 5, Table S1). *C. caretta* eDNA concentrations were higher at sites where sand samples were collected less than 12 h post-maternal false crawl (Figure 4b) as opposed to >24 h later (Figure 4a). This suggests that sand sampled closer to the sand disturbance event yields more sea turtle eDNA (although animal number and activity level are confounding factors) (Thalinger et al., 2021). However, sufficient sea turtle eDNA is still detectable and quantifiable many hours after the sand disturbance event (Figure 4a). Importantly, this fact benefits nesting-related field studies over large areas (nesting beaches) where it is difficult to be present at the exact time as the target organism. At (maternal) false crawl sites (Figure 4a, b), *C. caretta* eDNA concentrations were statistically significantly higher (*t*-test: $t(257) = 2.487$, $p < .05$) on the inner part of the track where the cloaca would have made the most contact, as opposed to the outer edges of the tracks where the flippers would have made the most contact. *C. mydas* eDNA was also successfully amplified from all maternal nesting crawl cloaca samples but was less consistent in *C. mydas* maternal nesting crawl flipper samples (Figure 4c).

Interestingly, *C. caretta* eDNA was even recoverable at detectible levels from the crawl track of a single hatchling (Figure 5a), demonstrating that even for very small animals shedding low quantities of DNA, positive species-specific detection is possible. *C. caretta* hatchlings weigh approximately 8–12 grams and cause minimal sand disturbance (Figure 5a). qPCR-based quantification revealed, as expected intuitively, more DNA was shed at sites where the hatchling halted its forward momentum and caused more disturbance while breathing ("Hatchling Crawl Paused For Breath" Figure 5a), than from crawl locations where the hatchling moved forward continuously ("Hatchling Crawl 1" and "Hatchling Crawl 2" Figure 5a) (Thalinger et al., 2021). These three hatchling crawls were all sampled from different points in the same crawl of one individual hatchling. From sand sampled from post-emergence nest spoil heaps (Figure 5b), sand corresponding to the nest surface had the highest levels of sea turtle eDNA, suggesting that the emerging hatchlings shed more DNA into their sand environment during their active crawl out of the nest, than the stationary eggs, sat dormant for weeks, deeper within the nest chamber.

To assess the viability of storage of sand compared with immediate (same day) eDNA extraction, we compared eDNA levels in biological replicates extracted either immediately or after three months storage at −20°C. Environmental DNA extracted from frozen sand also successfully amplified with the *C. caretta* qPCR assay (Table S2, Figure S6a) for all three "frozen" samples (amplification ratio of 1 in all six [100%] biological replicates). While this was a small sample size, it does appear that some eDNA was lost over time / via freeze-thaw. However, these results do suggest that prolonged storage of sand at −20°C prior to eDNA extraction is an acceptable approach if circumstances require it (if limited time/resource availability at the time of sample collection, or long-distance transport from field site to laboratory required).

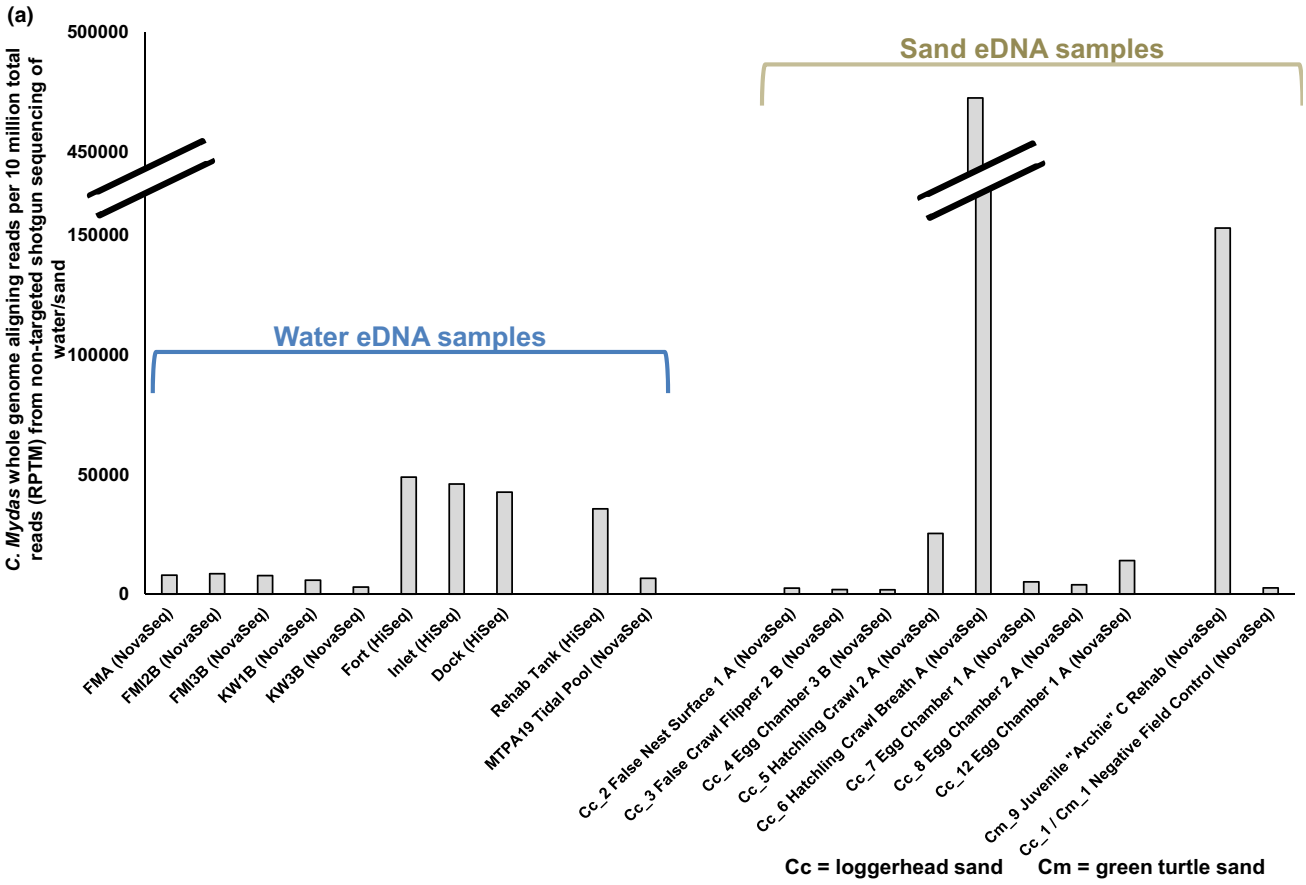
3.5 | Amplification ratio correlates to eDNA abundance – combined rehabilitation and field sample analysis

We next examined whether there was a correlation between amplification ratio and target eDNA abundance, utilizing all qPCR results from above. Interestingly, there was a strong positive correlation in all rehabilitation and wild samples, between average eDNA concentration (of sand and water eDNA) and amplification ratio (Pearson correlation coefficient: $R = 0.765$, $R^2 = 0.5852$, $p < .00001$), indicating, logically, that the higher the volume of target DNA present in the environmental sample, the more likely that each technical replicate will provide positive amplification (Figure 5c). This also suggests that low amplification ratios are indicative of target eDNA presence at low abundance.

3.6 | Shotgun sequencing-based detection of sea turtle DNA from environmental samples for population genetics – field trial

We next utilized shotgun sequencing to determine if sea turtle eDNA could be sequenced from selected water and sand samples. *C. mydas* aligning reads were detectable in all sequenced water and sand eDNA samples (Table S9, Figure 6a). There was a 259-fold difference between the field sample with the highest number of green turtle reads recovered (Cc_6 Hatchling Crawl Breath A, sand eDNA) and the sample with the lowest (Cc_4 Egg Chamber 3, sand eDNA) (Figure 6a). On average, more turtle aligning reads were recovered from sand eDNA samples (75,626 reads per 10 million total reads [RPTM]) than from water eDNA samples (21,334 RPTM), though both sample types had wide overlapping ranges (Figure 6a).

We next investigated whether sea turtle eDNA recovered from sand samples alone could be utilized for population genetics/genomics (geographic origin) and monitoring of nesting females and their hatchlings. Sequencing reads were aligned to reference mitochondrial genomes for each species. The mitochondrion was chosen as population genetics has been conducted on whole mitochondria and mitochondrial fragments for both species (Duchene et al., 2012; Komoroske et al., 2017; Shamblin, Bjorndal, et al., 2012; Shamblin et al., 2014, 2015; Shamblin, Bolten, et al., 2012), and as all clutch mates in nest samples share the same mitochondrial phylogeny (inherited from the mother). *C. mydas* and *C. caretta* mitochondrial DNA was detectable in all sequenced sea turtle rehabilitation and beach sand eDNA samples, with a range of 56 to 13,775 mitochondrial aligning reads (Table 1). The negative field control contained no sea turtle mitochondrial aligning reads. Only samples with more than 300 mitochondrion aligning reads were used for phylogenetic tree generation, haplo-grouping and haplotyping (Table 1). Consensus sequences were generated for these samples. Although they had no aligning sea turtle mitochondrial reads, both negative control sand samples were also used as controls in the consensus genome generation. In the absence of reads the consensus sequences for these


(a)
C. Mydas whole genome aligning reads per 10 million total reads (RPTM) from non-targeted shotgun sequencing of water/sand
500000
450000
150000
100000
50000
0
Water eDNA samples
Sand eDNA samples
FMA (NovaSeq)
FMI2B (NovaSeq)
FMI3B (NovaSeq)
KW1B (NovaSeq)
KW3B (NovaSeq)
Fort (HiSeq)
Inlet (HiSeq)
Dock (HiSeq)
Rehab Tank (HiSeq)
MTPA19 Tidal Pool (NovaSeq)
Cc_2 False Nest Surface 1 A (NovaSeq)
Cc_3 False Crawl Flipper 2 B (NovaSeq)
Cc_4 Egg Chamber 3 B (NovaSeq)
Cc_5 Hatchling Crawl 2 A (NovaSeq)
Cc_6 Hatchling Crawl Breath A (NovaSeq)
Cc_7 Egg Chamber 1 A (NovaSeq)
Cc_8 Egg Chamber 2 A (NovaSeq)
Cc_12 Egg Chamber 1 A (NovaSeq)
Cm_9 Juvenile "Archie" C Rehab (NovaSeq)
Cc_1 / Cm_1 Negative Field Control (NovaSeq)
Cc = loggerhead sand
Cm = green turtle sand

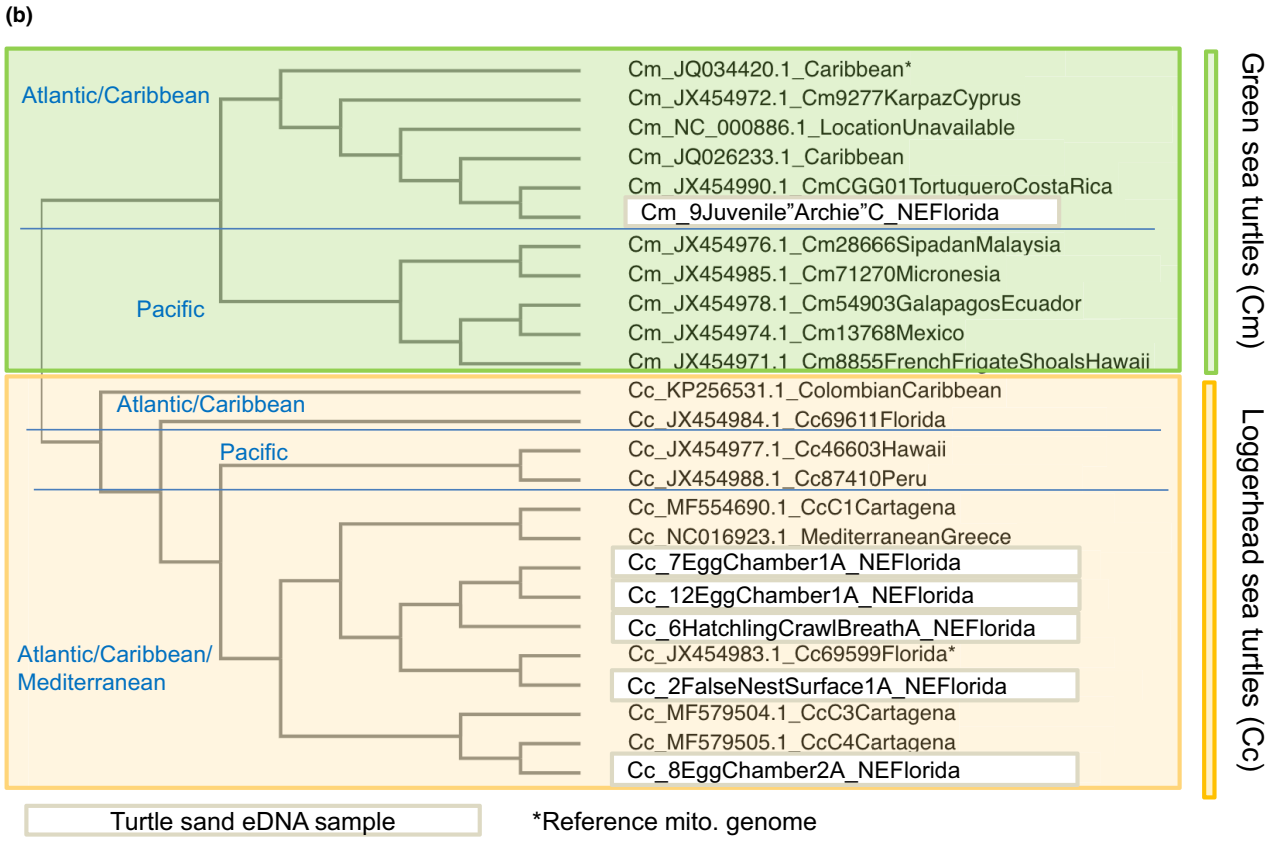

(b)
Atlantic/Caribbean
Pacific
Cm_JQ034420.1_Caribbean*
Cm_JX454972.1_Cm9277KarpazCyprus
Cm_NC_000886.1_LocationUnavailable
Cm_JQ026233.1_Caribbean
Cm_JX454990.1_CmCGG01TortugueroCostaRica
Cm_9Juvenile"Archie"C_NEFlorida
Cm_JX454976.1_Cm28666SipadanMalaysia
Cm_JX454985.1_Cm71270Micronesia
Cm_JX454978.1_Cm54903GalapagosEcuador
Cm_JX454974.1_Cm13768Mexico
Cm_JX454971.1_Cm8855FrenchFrigateShoalsHawaii
Green sea turtles (Cm)
Atlantic/Caribbean
Pacific
Atlantic/Caribbean/ Mediterranean
Cc_KP256531.1_ColombianCaribbean
Cc_JX454984.1_Cc69611Florida
Cc_JX454977.1_Cc46603Hawaii
Cc_JX454988.1_Cc87410Peru
Cc_MF554690.1_CcC1Cartagena
Cc_NC016923.1_MediterraneanGreece
Cc_7EggChamber1A_NEFlorida
Cc_12EggChamber1A_NEFlorida
Cc_6HatchlingCrawlBreathA_NEFlorida
Cc_JX454983.1_Cc69599Florida*
Cc_2FalseNestSurface1A_NEFlorida
Cc_MF579504.1_CcC3Cartagena
Cc_MF579505.1_CcC4Cartagena
Cc_8EggChamber2A_NEFlorida
Loggerhead sea turtles (Cc)
Turtle sand eDNA sample
*Reference mito. genome

FIGURE 6 Nontargeted Illumina shotgun sequencing of field eDNA from beach sand crawl/nest samples enables whole genome (nuclear and mitochondrial) sea turtle eDNA quantification and mitochondrial-based phylogenomic analysis. (a) Number of sand eDNA Illumina shotgun (no enrichment) sequenced reads from each water and sand sample which aligned to the *C. mydas* reference whole genome (Bentley et al., 2022; Wang et al., 2013). Numbers refer to sample ID numbers, not number of turtles. A–C refer to biological replicates. Note that all sand samples, with the exception of Cm_9 (rehab patient) and Cm/Cc_1 (negative field control), are from *C. caretta* samples, but no *C. caretta* reference whole genome was available. (b) Mitochondrion genome phylogenetic analysis (whole genome shotgun sequencing) of mitochondrial consensus genomes from our *C. mydas* and *C. caretta* sand eDNA samples, which had mitochondrial aligning reads above the cut off threshold (Table 1), compared with all *C. mydas* (10) and *C. caretta* (9) mitochondrion genomes available in GenBank (GenBank accession numbers: JQ034420.1, JQ026233.1, JX454971.1, JX454972.1, JX454974.1, JX454976.1, JX454978.1, JX454985.1, JX454990.1, NC_000886.1, JX454983.1, JX454977.1, JX454984.1, JX454988.1, KP256531.1, MF554690.1, MF579504.1, MF579505.1, NC_016923.1). Numbers refer to sample ID numbers, not number of turtles. A–C refer to biological replicates. Note the two mitochondrial genomes used as reference genomes for read alignment are highlighted by an asterisk (*). Our turtle sand eDNA samples are highlighted by white boxes. Each sample/mitochondrion label includes species, accession number, study specific number/name (when available) and geographic location (when available)

samples aligned directly with the chosen reference mitochondrial genome. All existing green and loggerhead sea turtle whole mitochondrial genomes currently available (accessed August 2021) in the NCBI GenBank public repository were used for phylogenetic reconstruction. The consensus mitochondrion sequences from sea turtle sand eDNA samples were divergent from the references they had been aligned to, and all grouped most closely with previously published mitochondrial genome sequences from the Atlantic/Caribbean, with none of our Florida samples grouping with Pacific mitochondrial sequences (Figure 6b). This confirms that even in the absence of target enrichment prior to sequencing, sea turtle genetic analyses can be conducted from sand sampling alone.

We next assessed smaller mitochondrial fragments commonly employed to haplotype sea turtles (LaCasella et al., 2007; Naro-Maciel et al., 2008; Nielsen et al., 2011; Shamblin, Bjorndal, et al., 2012; Shamblin et al., 2014, 2015; Shamblin, Bolten, et al., 2012). For all six samples their specific haplogroup could be called and their haplotype could be narrowed down (Table 1). However, coverage of the haplotype defining reads was not sufficient to call a single haplotype for each sample (Table 1). This is not surprising as these were metagenomics sand samples with no sea turtle enrichment prior to sequencing. Samples were called as haplotypes based only on haplotype defining sequence positions which were sufficiently covered by reads. From 34 defined loggerhead and 19 green turtle haplotypes, two samples (one green and one loggerhead) were narrowed down to three possible haplotypes each, with the range for all samples being 3 to 11 remaining haplotype possibilities (Table 1). Based on the available reads alone, every remaining haplotype narrowed down by the sequencing is known to occur in the nesting populations of the southeast US (including the regions containing the beaches sampled from for this study [Figure S1]), with available reads being sufficient to exclude haplotypes which have only been reported in more distant global locations (Table 1) (LaCasella et al., 2007; Naro-Maciel et al., 2008; Nielsen et al., 2011; Shamblin, Bjorndal, et al., 2012; Shamblin et al., 2014, 2015; Shamblin, Bolten, et al., 2012). While this is an expected result given the nesting locations sampled, it validates the capacity of eDNA to differentiate and identify (individually and geographical origin) turtles from sand samples alone. To further validate the haplotype calls from our nontargeted shotgun sequencing, we haplotyped green turtle rehabilitation patient "Archie" from archived nontumor tissue samples from our sea turtle fibropapillomatosis research. Conventional PCR followed by Sanger sequencing of patient "Archie" skin tissue extracted DNA (conducted after haplotyping from shotgun sequencing had been completed) confirmed that this individual was haplotype CM-A1.1, one of the 3 possible haplotypes independently narrowed down to from the nonenriched shotgun sequencing of the Archie sand sample (Table 1) (Sanger sequencing result was deposited at NCBI's Genbank under Accession no. ON093056). This supports the ability of eDNA sampling from sand samples to correctly haplogroup and haplotype sea turtles, especially if such techniques are in the future combined with targeted deep sequencing (prior enrichment of fragments of interest) or PCR approaches.

3.7 | Concurrent detection of sea turtles and their pathogens from beach sand eDNA - rehabilitation and field trial

Given that eDNA samples represent complex samples with DNA from a diverse number of organisms present, we next assessed whether we could concurrently detect a sea turtle pathogen from the same sand eDNA samples. Aligning the shotgun sequencing reads to the chelonid alphaherpesvirus 5 (ChHV5) reference genome (GenBank accession no.: HQ878327.2, Ackermann et al., 2012), confirmed that pathogens could be detected from the same sand samples (Figure 7a). High ChHV5 loads were recovered from the sand that fibropapillomatosis patient "Archie" had rested on (Figure 7a). ChHV5 is the presumptive etiological agent of the fibropapillomatosis tumor epizootic in green sea turtles. However, we also detected ChHV5 shed onto sand by the wild loggerhead hatchling, as it crawled from its nest to the ocean (Figure 7a). The presence of ChHV5 in crawl sand from this hatchling was confirmed by pathogen-specific qPCR (Figure S6b). Furthermore, even without any prior enrichment, sufficient ChHV5 reads were obtained from the fibropapillomatosis patient "Archie's" sand eDNA sample to enable viral variant calling (Figure 7b). This revealed that "Archie" was infected with a Florida variant of ChHV5, with the obtained sequence clustering with the

TABLE 1 Haplogroups and haplotypes of loggerhead (Cc) nesting females and emerging hatchlings, and a green (Cm) rehabilitation patient as determined from nontargeted shotgun sequencing of sand eDNA samples. All sequenced samples were generated using conventional PCR-based library preparation, with the exception of *Cc_12 Egg Chamber 1 A*. which was generated by PCR-free library prep, from the same eDNA sample as *Cc_7 Egg Chamber 1 A*. Accession numbers of all utilized public haplotype reference sequences can be found in Table S10

Sample	Absolute Concentration (pg/µl), species specific qPCR assay	Total aligning Reads to Cc or Cm Mitochondrion	Haplogroup	Haplotype (out of 34 Cc and 19 Cm possibilities) sand eDNA	Tissue haplotype confirmation with Sanger sequencing	Geographic origin
Cm_9 Juvenile "Archie" C	0.01374 *(Cm assay)*	770.5	A (Cm)	CM-A1.1, 1.2, 16.1	CM-A1.1	Florida east coast
Cc_2 False Nest Surface 1 A	0.00344 *(Cc assay)*	505	2 (Cc)	CC-A2.1, 2.2, 2.4, 2.11, 5.1, 7.1, 7.2, 13.1	-	US East Coast: 5/8 SEUS, 1/8 NWATL/CPV, 1/8 NWATL, 1/8 SEUS/Med
Cc_6 Hatchling Crawl Breath A	0.01814 *(Cc assay)*	13,775	2 (Cc)	CC-A2.1, 2.2, 2.4, 2.11, 5.1, 7.1, 7.2, 9.1, 10.4, 13.1, 42.1	-	US East Coast: 6/11 SEUS, 1/11 NWATL/CPV, 2/11 NWATL, 1/11 SEUS/Med, 1/11 QRMX
Cc_7 Egg Chamber 1 A	0.11013 *(Cc assay)*	6,915	2 (Cc)	CC-A2.1, 2.2, 2.4, 2.11, 5.1, 7.1, 7.2, 13.1	-	US East Coast: 5/8 SEUS, 1/8 NWATL/CPV, 1/8 NWATL, 1/8 SEUS/Med
Cc_12 Egg Chamber 1 A	0.11013 *(Cc assay)*	6,716.5	2 (Cc)	CC-A2.1, 2.2, 2.4, 2.11, 5.1, 7.1, 7.2, 9.1, 13.1, 42.1	-	US East Coast: 5/10 SEUS, 2/10 NWATL, 1/10 SEUS/MED, 1/10 NWATL/CPV, 1/10 QRMX
Cc_8 Egg Chamber 2 A	0.11007 *(Cc assay)*	353	1 (Cc)	CC-A1.1, 1.4, 27.1	-	US East Coast/north Florida (CAN): 2/3 SEUS, 1/3 NWATL/CPV
Cc_3 False Crawl Flipper 2 B	0.00069 *(Cc assay)*	55.5	Read number below cutoff threshold			
Cc_4 Egg Chamber 3 B	0.10286*(Cc assay)*	107				
Cc_5 Hatchling Crawl 2 A	0.00357 *(Cc assay)*	71				
Cm_1 Negative Field Control	0 *(Cc and Cm assay)*	0				
Cc_1 Negative Field Control	0 *(Cc and Cm assay)*	0				

closely related Florida variants A to C (Figure 7b). Unfortunately, the hatchling sand sample did not have sufficient ChHV5 reads to enable variant analysis. Subsequent, conventional PCR of patient "Archie" tumor samples confirmed the sand genomics result that Archie was infected with a Florida ChHV5 variant (Sanger sequencing result was deposited at NCBI's Genbank under Accession No. OM401718). Sand-based eDNA sampling has utility for simultaneously detecting sea turtle DNA but also that of their commensal microbes (metagenomics) and pathogens, enabling not just pathogen monitoring but also variant surveillance.

4 | DISCUSSION

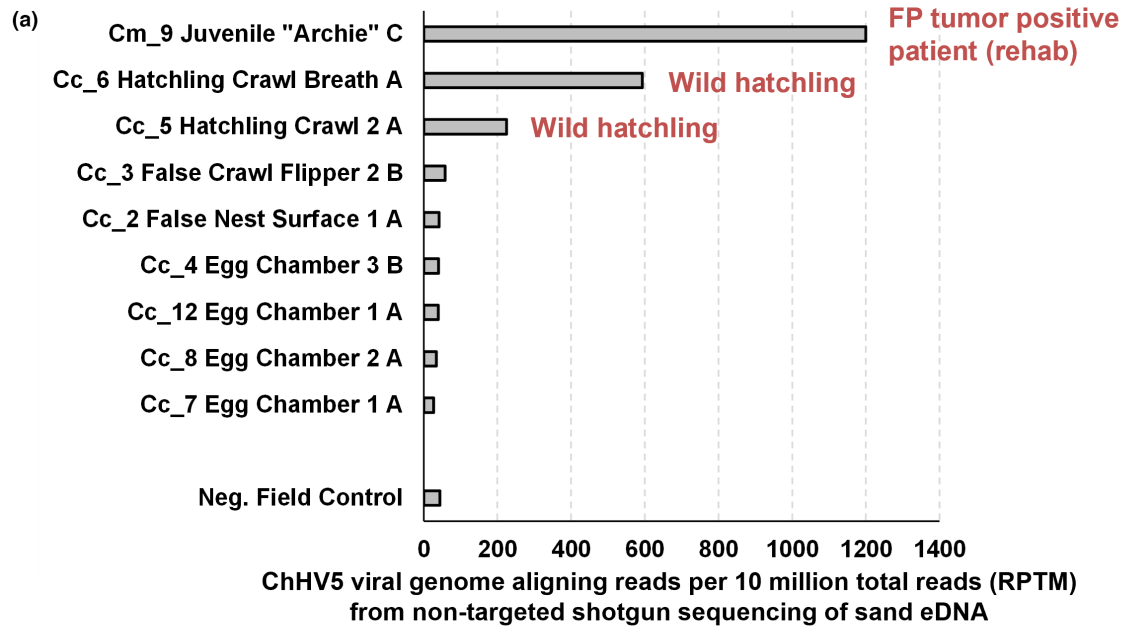
Within this study we successfully detected sea turtle species-specific eDNA in sand and water samples, in both rehabilitation and field settings. We also demonstrated the applicability of qPCR-based water eDNA approaches to detect the species-specific presence of sea turtle species in oceanic field trials. Furthermore, high-throughput sequencing-based eDNA approaches were applied to assess the genetics of vulnerable sea turtle populations from crawl tracks in beach sand left by nesting and hatching activity. Finally, these eDNA approaches could simultaneously detect and monitor the presence of sea turtle viral pathogens shed into the environment by these endangered species. These novel techniques can be applied to all seven sea turtle species around the world, as well as to the monitoring of numerous wildlife species and their pathogens, helping to pave the way for more targeted cost-effective eDNA-based monitoring and study of endangered wildlife and their disease threats.

4.1 | eDNA-based detection of sea turtles

Environmental DNA-based conservation approaches are redefining our ability to monitor and protect vulnerable species, and our study demonstrates the varied applications possible when such approaches are applied to the study and conservation of migratory species with complex life cycles, such as endangered sea turtles (Adams et al., 2019; Beng & Corlett, 2020; Farrell, Whitmore, et al., 2021; Farrell, Yetsko, et al., 2021; Lacoursiere-Roussel et al., 2016, 2018). This study has demonstrated the utility of species-specific *C. mydas* and *C. caretta* qPCR assays (Table S3) across a variety of environments from open-water to near-shore reefs and nesting beach sand. These sensitive assays detected sea turtle eDNA even from low abundance open water sites and from single hatchling crawls (*C. caretta* hatchlings weighing 8–12 g). All negative controls in this study (negative field controls and qPCR no template controls) were negative for sea turtle eDNA amplification, highlighting the specificity of the assays and the lack of contamination from all points in the collection, transport and laboratory process. Shotgun sequencing of the negative control field samples also failed to detect any sea turtle mitochondrial eDNA, while a limited number of reads from these samples did align to the 2.24 Gb entire green sea turtle reference genome this is probably due to conserved sequences not of sea turtle origin within these complex metagenomic samples. In addition to sand and water sampling, our results also indicate that marine leeches alone could be used to detect the species last fed upon, as an alternative nonvisual means of assessing sea turtle species range, and potentially enable sea turtle haplotyping from leech blood pellets. Furthermore, we revealed the ability to collect data for population genetics studies through shotgun sequencing or conventional PCR of eDNA extracted from sand crawl samples. These approaches open a variety of conservation and health applications for sea turtle eDNA, from surveying population range shifts, to simultaneous host and pathogen monitoring, to conducting noninvasive population genetics on nesting females and hatchlings (Farrell, Whitmore, et al., 2021; Farrell, Yetsko, et al., 2021; Yetsko et al., 2021). We previously successfully employed the *C. mydas* assay in a rehabilitation setting to correlate sea turtle viral shedding with fibropapillomatosis tumor burden and the shedding of host turtle eDNA in patient tanks (Farrell, Whitmore, et al., 2021; Yetsko et al., 2020). Here we applied the same *C. mydas* assay but to a wild open-water setting (as opposed to enclosed rehabilitation tanks) and successfully detected green sea turtles at wild study sites not only when a visual sighting of a turtle was possible at the time of water sample collection, but also at sites known to be sea turtle habitats but where no visual confirmation of a sea turtle was made at the time of surveying/sample collection (Figure 2c). Therefore, these optimized eDNA-based methods can complement traditional labour- and cost-intensive visual observation and capture-based surveys in the wild (Miaud et al., 2019; Valentini et al., 2016). Future adoption of eDNA-based monitoring is particularly important in light of the growing anthropogenic threats faced by sea turtles, including habitat disturbance/destruction, and tracking likely sea turtle range and habitat shifts thought to be occurring due to the climate crisis (Hamabata et al., 2020; Jensen et al., 2019; Mashkour et al., 2020). Rather than SYBR-based qPCR assays concurrently developed for green turtle water eDNA detection (Harper et al., 2020), this study (as well as our eDNA-focused sea turtle viral paper [Farrell, Yetsko, et al., 2021; Yetsko et al., 2021]) utilized more specific probe-based qPCR (for green and loggerhead turtles).

4.2 | eDNA approaches for population range and abundance estimates

We identified a positive correlation between *C. mydas* and *C. caretta* eDNA quantity, and known species abundance, in a controlled rehabilitation water setting. The assays reported here are immediately suitable for use in eDNA-based sea turtle range/presence and absence studies. Achieving accurate abundance estimates from eDNA data may require further refinement, although it should be noted that current abundance estimates based on visual sightings, mark/recapture or nesting female counts are far from ideal or absolutely accurate. Such estimates can be heavily biased and prone to error due to the inaccuracy of traditional abundance-estimated methods – human error of missed sightings or photograph capture/


(a)
Cm_9 Juvenile "Archie" C
Cc_6 Hatchling Crawl Breath A
Cc_5 Hatchling Crawl 2 A
Cc_3 False Crawl Flipper 2 B
Cc_2 False Nest Surface 1 A
Cc_4 Egg Chamber 3 B
Cc_12 Egg Chamber 1 A
Cc_8 Egg Chamber 2 A
Cc_7 Egg Chamber 1 A
Neg. Field Control
FP tumor positive patient (rehab)
Wild hatchling
Wild hatchling
0
200
400
600
800
1000
1200
1400
ChHV5 viral genome aligning reads per 10 million total reads (RPTM) from non-targeted shotgun sequencing of sand eDNA

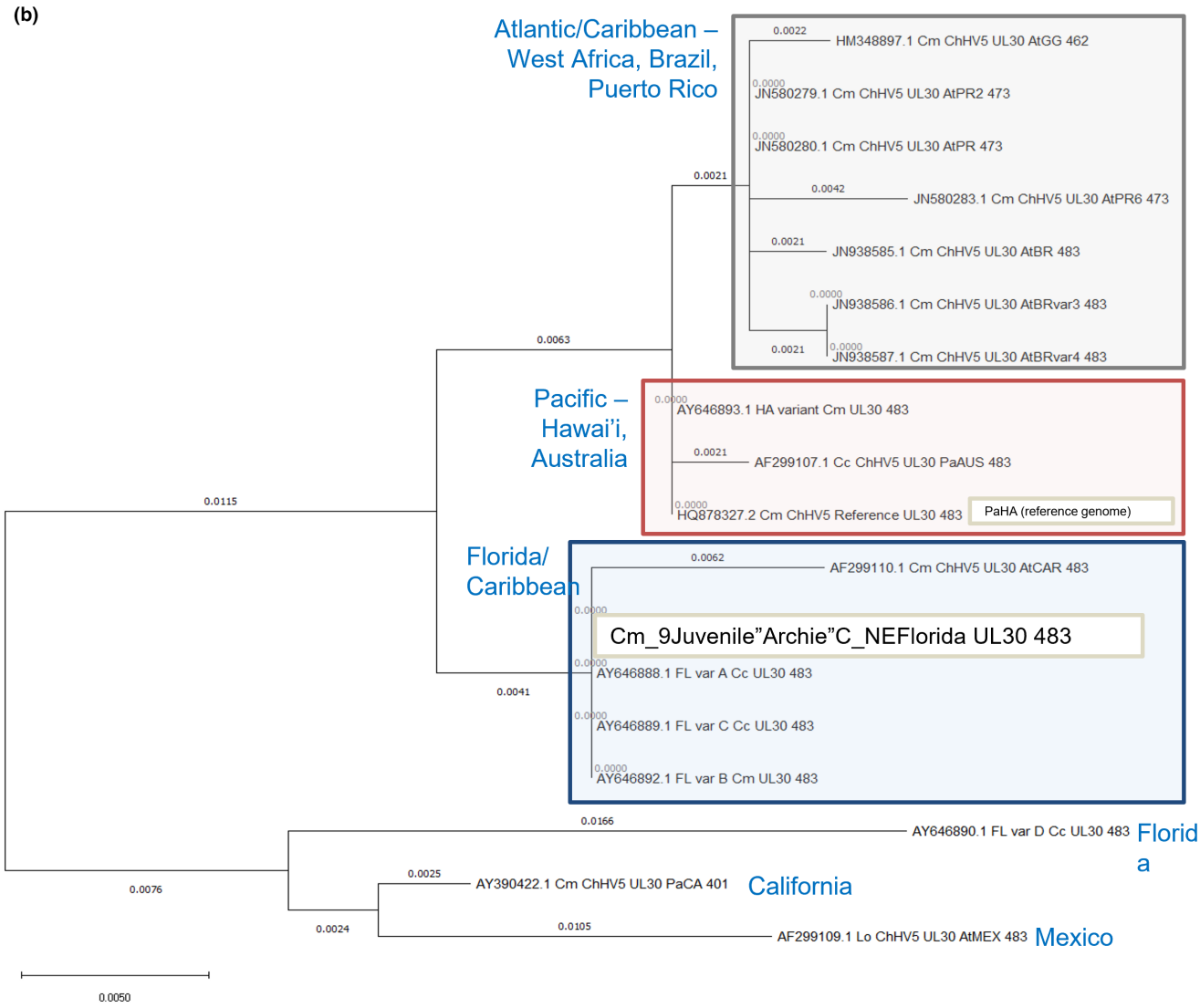

(b)
Atlantic/Caribbean – West Africa, Brazil, Puerto Rico
HM348897.1 Cm ChHV5 UL30 AtGG 462
JN580279.1 Cm ChHV5 UL30 AtPR2 473
JN580280.1 Cm ChHV5 UL30 AtPR 473
JN580283.1 Cm ChHV5 UL30 AtPR6 473
JN938585.1 Cm ChHV5 UL30 AtBR 483
JN938586.1 Cm ChHV5 UL30 AtBRvar3 483
JN938587.1 Cm ChHV5 UL30 AtBRvar4 483
Pacific – Hawai'i, Australia
AY646893.1 HA variant Cm UL30 483
AF299107.1 Cc ChHV5 UL30 PaAUS 483
HQ878327.2 Cm ChHV5 Reference UL30 483
PaHA (reference genome)
Florida/ Caribbean
AF299110.1 Cm ChHV5 UL30 AtCAR 483
Cm_9Juvenile"Archie"C_NEFlorida UL30 483
AY646888.1 FL var A Cc UL30 483
AY646889.1 FL var C Cc UL30 483
AY646892.1 FL var B Cm UL30 483
AY646890.1 FL var D Cc UL30 483
Florida
AY390422.1 Cm ChHV5 UL30 PaCA 401
California
AF299109.1 Lo ChHV5 UL30 AtMEX 483
Mexico
0.0050

FIGURE 7 Nontargeted Illumina shotgun sequencing of sea turtle eDNA from sand samples enables ChHV5 pathogen quantification and viral variant analysis. (a) ChHV5 viral genome aligning reads from nontargeted shotgun sequencing of wild sand samples using whole genome shotgun sequencing. Numbers refer to sample ID numbers, not number of turtles. A–C refer to biological replicates. These are the same sand samples used for sea turtle-related analysis above. (b) ChHV5 variant phylogenetic analysis from the patient "Archie" rehabilitation sand eDNA sample. Phylogenetic analysis of partial ChHV5 UL30 gene (483 bp) from the ChHV5 consensus sequence generated from the "Archie" sand sample's ChHV5 aligning reads (obtained by nontargeted Illumina shotgun sequencing). Numbers refer to sample ID numbers, not number of turtles. a–c refer to biological replicates. The ChHV5 UL30 sequence obtained from the "Archie" sand sample was compared with published ChHV5 UL30 sequences from NCBI. All sample names in the phylogenetic tree begin with that sequence's NCBI accession number and end with the length of the sequence in base pairs (note there are some slight length discrepancies between samples based on deposited sequence length). The sea turtle species ChHV5 was sequenced from is provided as follows Cm – *Chelonia mydas* (green), Cc – *Caretta caretta* (loggerhead), Lo – *Lepidochelys olivacea* (olive ridley). The "Archie" ChHV5 sequence obtained from sand eDNA is highlighted by a grey box. The ChHV5 reference genome sequence, which the sand sample was aligned to, is also highlighted by a grey box

identification of sea turtles can result in potentially underestimated population abundances. Similarly, estimating population size based on nest counts is complicated by its complete omission of all males and nonsexually mature females, by females having different internesting periods (ranging from nesting every year to several years between nesting) and by the tendency to lay multiple nests per season, sometimes spread over wide geographic ranges. In comparison, eDNA estimates are entirely objective, cover all sexes and life-stages, and simply rely on the accurate collection of a water sample, not the accurate sighting of all possible elusive aquatic individuals within a study area (Casale & Ceriani, 2020; Dunstan et al., 2020; Laloe et al., 2020; Whiting et al., 2020). To the best of our knowledge, the data reported here is the first demonstration of eDNA-based qPCR assays to estimate sea turtle biomass abundance within a controlled marine setting. Previous eDNA-based studies have been limited to presence/absence, freshwater estimates, or unrelated marine taxa such as octopus (Harper et al., 2020). Future studies should focus on applying these methodologies to species quantification, tracking and monitoring-based projects of aquatic sea turtle populations (Gredzens & Shaver, 2020; Mettler et al., 2020; Mortimer et al., 2020; Pfaller et al., 2020). If abiotic factors are accurately accounted for, this methodology could be optimized to estimate ranges and the population abundance of specific sea turtle species in coastal waters. Environmental DNA monitoring data are increasingly being coupled with mathematical models for more robust population dynamics, range and abundance estimates, with varying success (Schmelzle & Kinziger, 2015; Keck et al., 2022; Martel et al., 2020; Sales et al., 2020; Burian et al., 2021). For example, occupancy models that estimate occurrence and detection probability indicated that eDNA detection was positively related to an index of target species density (Strickland & Roberts, 2018).

4.3 | eDNA approaches to sea turtle population genetics

Current population genetics approaches require invasive tissue sampling or blood draws – for sea turtles this frequently is conducted during nesting, physically interacting with nesting females and probably inducing additional stress – alternatively one egg needs to be sacrificed per clutch to obtain maternal DNA without direct tissue/blood sampling (Adams et al., 2019; Calmanovici et al., 2018; Gadagkar et al., 2005; Gatto et al., 2018; Jensen et al., 2019; Komoroske et al., 2018; Long & Azmi, 2017; Shamblin et al., 2011). These approaches limit investigators to sampling only when physically present during nesting events, or immediately after egg laying. We have demonstrated that genetic material can be recovered for population genetics studies noninvasively from sand the turtle has passed over/interacted with, and hours to days after the nesting event occurred, without ever needing to physically observe or interact with nesting females or hatchlings during crucial life events (nesting and nest emergence). Of all the sand samples analysed (rehabilitation and field setting) the greatest quantity of sea turtle eDNA obtained was from a nest surface sand sample taken after hatchling emergence (Figure 5b). While it has been hypothesized that sand as a substrate could result in higher detection probability due to its lower DNA binding capacity (as opposed to topsoil or clay that have a larger number of DNA binding sites), no studies have yet elucidated a definitive comparison between the substrate types (Buxton et al., 2017). However, finer substrate sand has been shown to retain more eDNA than courser substrate gravel (Shogren et al., 2017). With nontargeted sequencing we recovered sufficient sea turtle DNA from sand to haplogroup individuals. All haplotypes identified from sand samples are known to predominantly occur in populations in the Southeast US, our study location (Shamblin, Bjorndal, et al., 2012; Shamblin et al., 2014, 2015; Shamblin, Bolten, et al., 2012). Coupled with pre-enrichment of target regions of interest or conventional PCR-based approaches, any genetic study currently requiring tissue/blood should be feasible from sand eDNA (i.e., haplotyping or microsatellite-based fingerprinting), similar to how haplotyping of whale sharks is now possible from just seawater eDNA (Dugal et al., 2021). Given the proof-of-principle results revealed by nontargeted metagenomic shotgun sequencing, the development of cost-effective targeted enrichment (prior exome enrichment, or targeted mitochondrial and microsatellite marker regions) next-generation sequencing eDNA approaches for sea turtle population genomics should be a key priority of future studies. Sand eDNA also benefits from being more straightforward than conventional invasive sampling approaches, enabling samples to be collected by networks of citizen-scientists/nesting patrol members, as utilized for this study.

4.4 | eDNA approaches for pathogen monitoring and surveillance

In addition to the sea turtle species-specific research possible with the methodologies established in this study, it is also possible to extend these genetic analysis tools further to include the investigation and monitoring of threats to these vulnerable animals (Amarasiri et al., 2021; Diaz-Ferguson & Moyer, 2014; Farrell, Yetsko, et al., 2021; Huver et al., 2015; Miaud et al., 2019; Sengupta et al., 2019). Given that eDNA extraction recovered DNA from all organisms/pathogens present (shed DNA or microbes themselves) there is significant potential to simultaneously monitor wildlife and their pathogens from the same eDNA sample (Alfaro-Nunez et al., 2014; Amarasiri et al., 2021; Duffy & Martindale, 2019; Duffy et al., 2018; Farrell, Whitmore, et al., 2021; Patricio et al., 2012). Indeed, some of the rehabilitation water and sand eDNA samples utilized for sea turtle detection in the present study, were also simultaneously utilized to quantify a viral pathogen of sea turtles (Farrell, Whitmore, et al., 2021). This pathogen is a turtle-specific ChHV5 virus and can result in the tumor disease, fibropapillomatosis (FP) (Chaves et al., 2017; Farrell et al., 2018; Farrell, Yetsko, et al., 2021; Page-Karjian et al., 2015, 2017; Work et al., 2015, 2020; Yetsko et al., 2021). We previously adapted the eDNA methodology described here to successfully detect, quantify, and temporally track the concentration of ChHV5 DNA in water (qPCR and shotgun sequencing) and sand (qPCR only) samples in a controlled rehabilitation setting (Farrell, Yetsko, et al., 2021; Yetsko et al., 2021). Here we report ChHV5 variant calling was possible from nontargeted shotgun sequencing of a juvenile *C. mydas* FP-afflicted patient sand sample. Moving beyond such captive settings, we also report here that ChHV5 DNA was detected (by qPCR and shotgun sequencing) in the crawl sand of an FP-free *C. caretta* hatchling, as it made its way from its nest to the ocean. The level of ChHV5 DNA recovered correlated to the level of *C. caretta* DNA recovered, with more sea turtle and virus eDNA being present in samples from an active breath site, compared with a continuous forward motion crawl sample. It was thought that ChHV5 was only horizontally transmitted when juvenile turtles recruited to nearshore feeding grounds (Jones et al., 2020). The detection of ChHV5 in fresh hatchling crawl sand eDNA samples reported here, suggests that ChHV5 may actually be vertically transmitted from mother to offspring, or horizontally transmitted before a hatchling reaches the ocean. This finding supports our previous detection of ChHV5 in tissue samples from a limited number of hatchlings (Farrell, Yetsko, et al., 2021).

Taken together, we report the successful application of eDNA-based molecular and genetics-based approaches, that have been adapted for the monitoring of human pathogens and other wildlife species, to advance the field of sea turtle conservation by providing novel noninvasive, accurate, cost-effective and efficient monitoring tools (Adams et al., 2019; Ahmed et al., 2020; Doyle & Uthicke, 2021; Ficetola et al., 2008; Farrell, Whitmore, et al., 2021; Miaud et al., 2019; Qu & Stewart, 2019).

4.5 | Conclusion

The results of this study provide support for the use of eDNA (qPCR and deep sequencing-based analysis) to detect, monitor, identify (geographic origin/individual fingerprint) and ultimately help conserve threatened sea turtle species. We have successfully detected and quantified *C. mydas* and *C. caretta* eDNA in sand and water samples in rehabilitation and wild settings. We have also shown that the population genetic structure of sampled turtles can be identified from noninvasive sand eDNA sampling alone as can monitoring of sea turtle pathogens, such as the putative oncogenic pathogen ChHV5. These approaches open the door to large-scale cost-effective eDNA-based population genetics studies with targeted sequencing approaches. They have potential utility not just for the two sea turtle species studied here, but for all seven sea turtle species and countless other wildlife inhabiting aquatic and terrestrial environments. While further targeted optimizations are possible, the methodologies described in this study could vastly exceed traditional observation and capture-based sea turtle monitoring methods and enable more sites to be studied without increasing the effort required. The time, cost and sampling effectiveness of these approaches make the study of sea turtles in more remote or impoverished sites far more amenable. Wider adoption of eDNA-based monitoring by conservationists and researchers will help to improve understanding and better combat wildlife population changes induced by anthropogenic and natural threats, such as climate change and disease.

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tissue sampling from the University of Florida's Institutional Animal Care and Use Committee (IACUC). Rehabilitation and nest patrol activities are conducted under FWC permit numbers MTP-21–228, MTP-21–103, MTP-21–084, MTP-21–029, MTP-21–041, MTP-21–140, MTP-21–023, MTP-21–046 and MTP-21–101 (rehabilitation and conservation activities were in no way impacted by this study). The Inwater Research Group conduct in-water sea turtle monitoring surveys under NMFS permit numbers 19528 and 16598 and FWC permit numbers MTP-18–125 and MTP-18–139. The Florida Hawksbill project conducts conduct in-water sea turtle monitoring surveys under NMFS permit number 22988 and FWC permit number MTP-21–077.

CONFLICT OF INTERESTS

The authors declare no competing interests. Additionally, the funding agencies had no role in the conceptualization, design, data collection, analysis, decision to publish, or preparation of the manuscript. L. Wood now also works for one of the funders, the National Save The Sea Turtle Foundation, Inc., but his role in this study was prior to taking up appointment with the funder.

AUTHOR CONTRIBUTIONS

David J. Duffy, Jessica A Farrell and Liam Whitmore designed the project and David J. Duffy supervised the project. Jessica A Farrell, David J. Duffy, Liam Whitmore, Narges Mashkour, and Kelsey Yetsko generated the data. Devon R Rollinson Ramia, Rachel S. Thomas, Catherine B Eastman, and Brooke Burkhalter provided veterinary care to the rehabilitation patients within this study and facilitated rehabilitation sample collection and tissue sampling. Nancy Condron, Cody Mott, Larry Wood, Bette Zirkelbach, Lucas Meers, Pat Kleinsasser, Sharon Stock, Elizabeth Libert, Richard Herren, Scott Eastman and David Godfrey assisted with field sample collection. Jessica A Farrell and David J. Duffy wrote the manuscript. All authors read and approved the final manuscript.

OPEN RESEARCH BADGES

This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at [provided https://www.ncbi.nlm.nih.gov/bioproject/PRJNA449022].

DATA AVAILABILITY STATEMENT

All sequenced samples including raw reads are deposited in NCBI (https://www.ncbi.nlm.nih.gov/) under BioProject ID: PRJNA449022(https://www.ncbi.nlm.nih.gov/bioproject/PRJNA449022). The Sanger sequenced ChHV5 PCR product for viral variant identification is deposited in NCBI's Genbank under accession number OM401718. The Sanger sequenced mitochondrial PCR product for green turtle haplotyping is deposited in NCBI's Genbank under accession number ON093056. All other data is available in the Supporting Information tables.

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