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ORIGINAL ARTICLE OPEN ACCESS

Environmental DNA Biosurveillance of Chelonid Herpesvirus 5 (ChHV5) in Endangered Sea Turtles Around Singapore

Yin Cheong Aden Ip[1] | Li Ying Tan[1] | Chee Wai Lee[1] | Jing Chen[1] | Ravendrakumar Shanmugavelu Balasubramaniam[1] | Muhammad Faizal Sim[1] | Collin Hor Yee Tong[2] | Jerome Yong[2] | Yifan Wang[1] | Xuan Hui Teo[1] | Christine H. E. Lee[1] | Zi Yan Brian Tan[1] | Charlene Judith Fernandez[1] | Siow Foong Chang[1] | Kenneth B. H. Er[3]

[1]Animal & Veterinary Service, National Parks Board, Singapore, Singapore | [2]Coastal & Marine Branch, National Parks Board, Singapore, Singapore | [3]National Parks Board Headquarters, Singapore, Singapore

Correspondence: Zi Yan Brian Tan (brian_tan@nparks.gov.sg)

Received: 4 August 2025 | **Revised:** 29 January 2026 | **Accepted:** 26 March 2026

Keywords: chelonid herpesvirus 5 | endangered species | environmental DNA | green turtles | hawksbill turtles | pathogen genomics | phylogenetics

ABSTRACT

Sea turtle fibropapillomatosis (FP) is a potentially debilitating and neoplastic disease closely associated with the aetiological agent chelonid herpesvirus 5 (ChHV5). FP occurs in all endangered sea turtle species across most oceanic basins, with incidences rising rapidly in many geographic locations. Although FP is an emerging disease threat, the prevalence of FP and ChHV5 in turtle populations around Southeast Asia remains relatively understudied, underscoring the need for effective disease monitoring and surveillance strategies. To address this gap, our study reports the first near-complete ChHV5 genome from Southeast Asia, which was obtained from an FP-afflicted turtle stranded in Singapore waters in 2022. Along with the host mitochondrial genome, the viral genome provides a key reference point for ChHV5 distribution and host population origins from the region. To further enhance monitoring efforts, we also explored the application of environmental DNA (eDNA) methods as a non-invasive biosurveillance tool for detecting ChHV5 DNA in seawater and sediment. Using real-time PCR, ChHV5 DNA was successfully detected in rehabilitation tank water housing the afflicted turtle, with viral abundance increasing after turtle reintroduction into the tank. This observation is consistent with direct viral shedding as a potential ChHV5 transmission route. Lastly, the feasibility of detecting ChHV5 DNA from sediment samples using real-time PCR was assessed through a spike-and-recovery experiment. This method was subsequently applied to sediment samples collected from hawksbill turtle nesting sites around Singapore, with no positive detections observed. Overall, the eDNA methods and next-generation sequencing approaches described herein establish a baseline for ChHV5 biosurveillance, providing important reference data to inform future sea turtle monitoring efforts in Southeast Asia.

1 | Introduction

Sea turtle populations inhabiting oceans worldwide are facing an imminent threat of extinction due to a complex combination of environmental and anthropogenic factors that warrant coordinated intervention efforts. This threat is particularly evident in highly urbanized coastal regions such as Singapore, where limited natural habitats nonetheless retain conservation value by supporting regional sea turtle populations. Despite Singapore's dense coastal development and lack of natural shorelines, its waters still serve as foraging grounds for green turtles (*Chelonia mydas*) and hawksbill turtles (*Eretmochelys imbricata*), which are classified as "endangered" and "critically endangered" respectively (Seminoff 2023; Mortimer and

Donnelly 2008). Additionally, several beaches and offshore islands around Singapore provide important nesting grounds for hawksbill turtles. While local conservation efforts, such as a turtle hatchery, are already underway to protect these charismatic marine animals, improving the long-term survivability of sea turtles depends on addressing broader challenges. These include nesting ground loss due to climate-driven beach erosion and inundation (Fuentes et al. 2011), skewed hatchling sex ratios due to rising sand temperatures (Laloë et al. 2016), poaching (Tomillo et al. 2008; Joseph et al. 2019), and the threat of emerging infectious diseases like fibropapillomatosis (FP) (Farrell et al. 2021; Robben et al. 2023; Manes et al. 2023).

FP is a neoplastic disease documented in all seven sea turtle species, with green turtles being the most susceptible (Jones et al. 2016; Dujon et al. 2021). FP often manifests as a debilitating condition characterized by tumorous growths on the turtle's skin, eyes, and internal organs. These tumors can impede natural behaviors such as swimming, predator evasion, and feeding, and can ultimately result in mortality (Jones et al. 2016). At the population level, long-term studies from Florida and Akumal Bay show high or increasing FP prevalence, highlighting the disease as an ecological signal that may indicate environmental stress or reflect potential impacts on population health (Foley et al. 2005; Muñoz Tenería et al. 2022; Kelley et al. 2022). The putative aetiological agent of FP has been identified as chelonid herpesvirus 5 (ChHV5) based on transmission studies and molecular detection methods, although its causative role has not been confirmed through Koch's postulates (Quackenbush et al. 2001; Herbst et al. 2004; Work et al. 2017). FP occurrence also appears to be associated with several environmental and ecological triggers, and may not always be found in conjunction with ChHV5 detections (Ene et al. 2005; Morrison et al. 2018; Mashkour et al. 2021). This suggests that biosurveillance strategies are still crucial in monitoring the prevalence and distribution of both FP and ChHV5 in order to elucidate the factors contributing to disease spread.

In particular, there is a current lack of studies documenting the occurrence of FP and ChHV5 in Southeast Asia, and no published data exist yet from Singapore. However, reports of recent cases from neighboring regions such as Taiwan and Malaysian Borneo highlight the proximity of the virus to Singapore's coastal waters (Li et al. 2017; Loganathan et al. 2021; Robben et al. 2023). For instance, from a single study site in Malaysia, Robben et al. (2023) reported a 42.9% increase in the prevalence of ChHV5 in green turtles compared to previous sampling and provided new evidence of ChHV5 infections in hawksbill turtles in the region. Therefore, similar assessments of ChHV5 and FP risks are increasingly important in Singapore's local context. While sea turtles are regularly sighted around Singapore, efforts to systematically document these occurrences have only begun recently (Cham et al. 2025). Accordingly, there is a lack of ecological data and an absence of a local baseline for ChHV5 prevalence, which is compounded by the difficulty of monitoring these elusive and free-ranging animals. Traditional biological sampling at nesting sites may also cause undue stress to nesting mothers and emerging hatchlings (Farrell et al. 2022). Given these constraints, non-invasive environmental DNA (eDNA) sampling offers a promising alternative for monitoring and detecting ChHV5 in sea turtle populations.

Environmental DNA approaches have been increasingly applied for pathogen surveillance in both aquatic and terrestrial systems. These methods recover genetic material from microorganisms, sloughed cells, and free-floating nucleic acids present in environmental matrices such as sediment, water, and air, reducing the need for direct sampling from target animals (Bohmann et al. 2014; Bass et al. 2023). Analyses of environmental nucleic acids rely on molecular detection techniques such as real-time PCR and high-throughput sequencing workflows like metabarcoding and metagenomics. These techniques have been used to monitor pathogens including bacteria, fungi, and parasites (Lo et al. 2023; Lastra González et al. 2021; Thomas et al. 2022), with recent studies also conducting more comprehensive assessments of pathogen communities (e.g., Sieber et al. 2024). In the context of sea turtle health, Farrell et al. (2021, 2022) successfully applied eDNA methods for ChHV5 viral DNA detection in both water and sediment samples. Farrell et al. (2021) showed that ChHV5 DNA could be readily detected in water collected from tanks housing turtles afflicted with FP, while the 2022 study further validated the use of eDNA methods for ChHV5 DNA detection in sediment samples collected from natural nesting sites. Together, these findings highlight the value of eDNA sampling for ChHV5 detection and represent a potential paradigm shift in evaluating the health of sea turtle populations. Notwithstanding this, these eDNA methods have yet to be applied for disease monitoring in endangered turtle species and populations around Singapore.

Our study therefore explores the application of eDNA methods and genomic sequencing to better assess the threat posed by ChHV5 and FP to Singapore's endangered sea turtles, using both passive and active biosurveillance approaches (Figure 1). We report three cases of FP in Singapore between 2020 and 2022: two from green turtle carcasses and one from a live green turtle admitted to a rehabilitation facility. Building on these cases, our study's objectives are to (1) generate genomic data of both the virus and host to place the Singapore cases in a broader global context, (2) apply eDNA methods to detect ChHV5 viral DNA in rehabilitation tank water, and (3) explore the feasibility of extending eDNA-based ChHV5 surveillance to sediment from hawksbill turtle nesting sites. Real-time PCR was used for preliminary screening of all tissue and eDNA samples, while high-throughput sequencing of the tissue samples provided a regional reference ChHV5 genome, alongside host mitochondrial data, for future phylogenetic studies. Collectively, these approaches establish a baseline for future ChHV5 monitoring efforts in Singapore, improve our understanding of the virus' occurrence in local sea turtle populations, and support the development of more effective conservation strategies in the future.

2 | Methods

2.1 | Sample Collection

2.1.1 | Tumor Tissue From Green Turtle Carcasses

In August 2020 and 2021, two adult green turtle carcasses in varying states of decomposition were discovered ashore at East Coast Park along the Singapore Straits. Both carcasses displayed multiple cutaneous tumors in various body locations, including

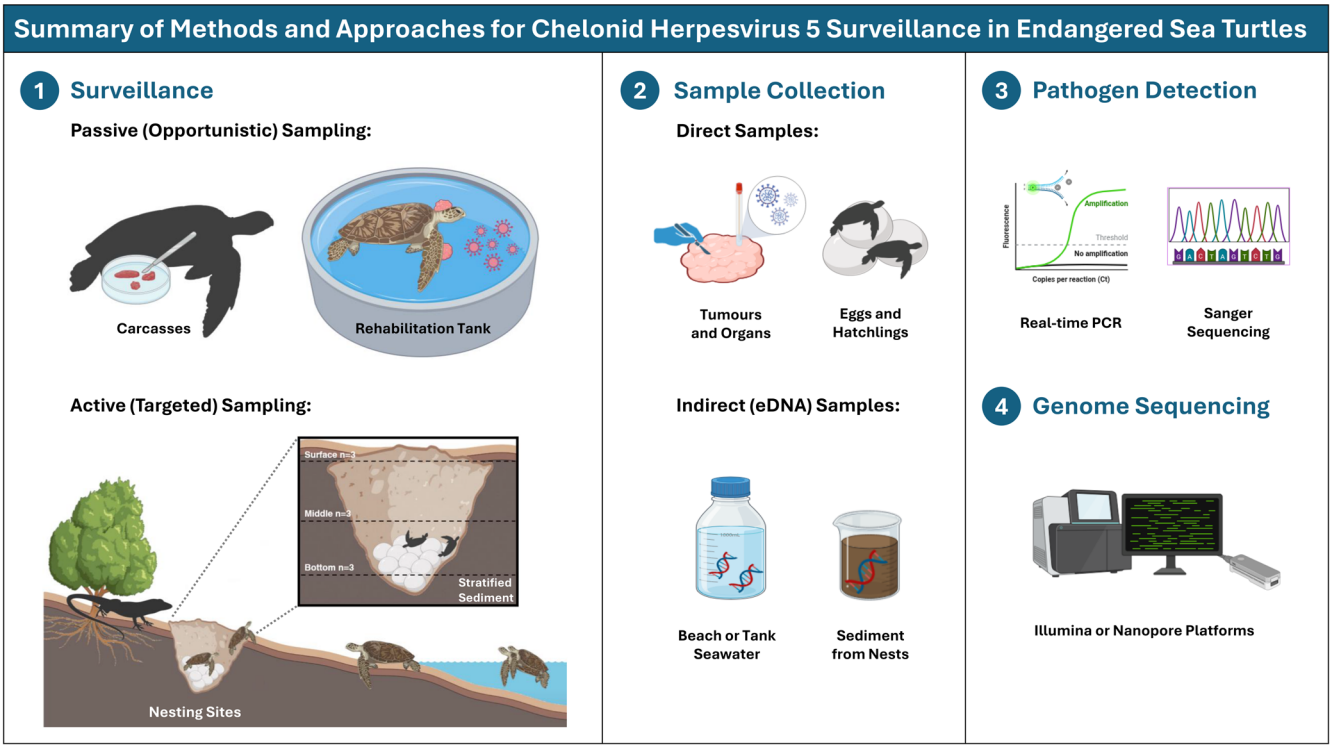

FIGURE 1 | Workflow overview for ChHV5 virus biosurveillance in Singapore's endangered sea turtles. The figure is divided into three main panels: (1) Surveillance approaches, (2) Sample collection, involving both direct and indirect (eDNA) sample types, and (3) Molecular detection and high-throughput whole-genome sequencing approaches.

the eyes, neck, and flippers, which is consistent with the clinical presentation of FP. Subsequently, the specimens were sent to the Centre for Animal and Veterinary Sciences (CAVS), National Parks Board, Singapore, for necropsy and microbiological analysis. The carcasses found in 2020 and 2021 were labeled as A6/8/20 and A6/8/21, respectively. For A6/8/20, internal tumors were sampled from the peritoneal lining and external tumors were sampled from the skin, and both the internal and external tumors were processed for virological testing. For A6/8/21, in addition to internal and external tumors, various organs (spinal cord, brain, heart, lung, trachea, stomach, intestine, muscle, kidney, spleen, and liver) were also sampled and processed for virological testing.

2.1.2 | Live Green Turtle Rehabilitation and Seawater eDNA Collection

In August 2022, a live adult green turtle was rescued by the public near Lazarus Island, which is an offshore island around Singapore. The turtle exhibited multiple tumors (refer to Figure S1) and was found stranded and unable to dive. Subsequently, the National Parks Board was alerted, and arrangements were made for the turtle to be sent to a rehabilitation facility. Tumor tissue samples were collected from the turtle's neck and left front flipper (refer to Figure S1), placed in sterile containers, and stored on ice immediately. Swab samples were also taken from the turtle's left eye, right eye, cloaca, throat, mouth, and two tumor wounds following tumor biopsy, yielding a total of 7 swabs across these anatomical sites. Each swab was placed in a tube containing 3 mL of viral transport medium (VTM) and stored on ice immediately.

The rehabilitation tank that housed the green turtle was cylindrical, approximately 1 m in radius and height, and held an estimated volume of 3,000 L. Seawater was collected from the surface of the tank in two batches using sterile 1-L Nalgene Wide-Mouth PPCO bottles (Fisher Scientific), for a total of 6 L and with no replicates. The first batch, which consisted of 4 L, was sampled ~30 min after the live turtle was removed from the tank for biopsy. The second batch, consisting of 2 L, was sampled 5 min after the turtle was reintroduced into the tank. To minimize the risk of contamination, all eDNA sampling equipment and gloves were disinfected with 10,000 ppm sodium hypochlorite and dried before use. Following collection, the water samples were immediately placed in a sterile cooler box and transported back to the laboratory for filtration and storage at −80°C. All tissue samples were also preserved at −80°C until further processing.

2.1.3 | Hawksbill Turtle Nest Sampling and Sediment eDNA Collection

A total of 12 hawksbill turtle nests were sampled around Singapore's mainland and offshore islands (Figure 2) during the nesting seasons (July to September) in 2022 and 2023, with 5 and 7 nests sampled in those years respectively. Freshly laid nests were marked with GPS coordinates, and subsequent visits were made between Days 48 to 52 of the incubation period to monitor the nests and hatchlings. In 2022, the nests were manually excavated 4 days after the hatchlings' departure for the collection of dead hatchlings, unhatched eggs, and sediment samples. Sediment from the bottom, middle, and surface of each nest was collected using sterile 15 mL falcon

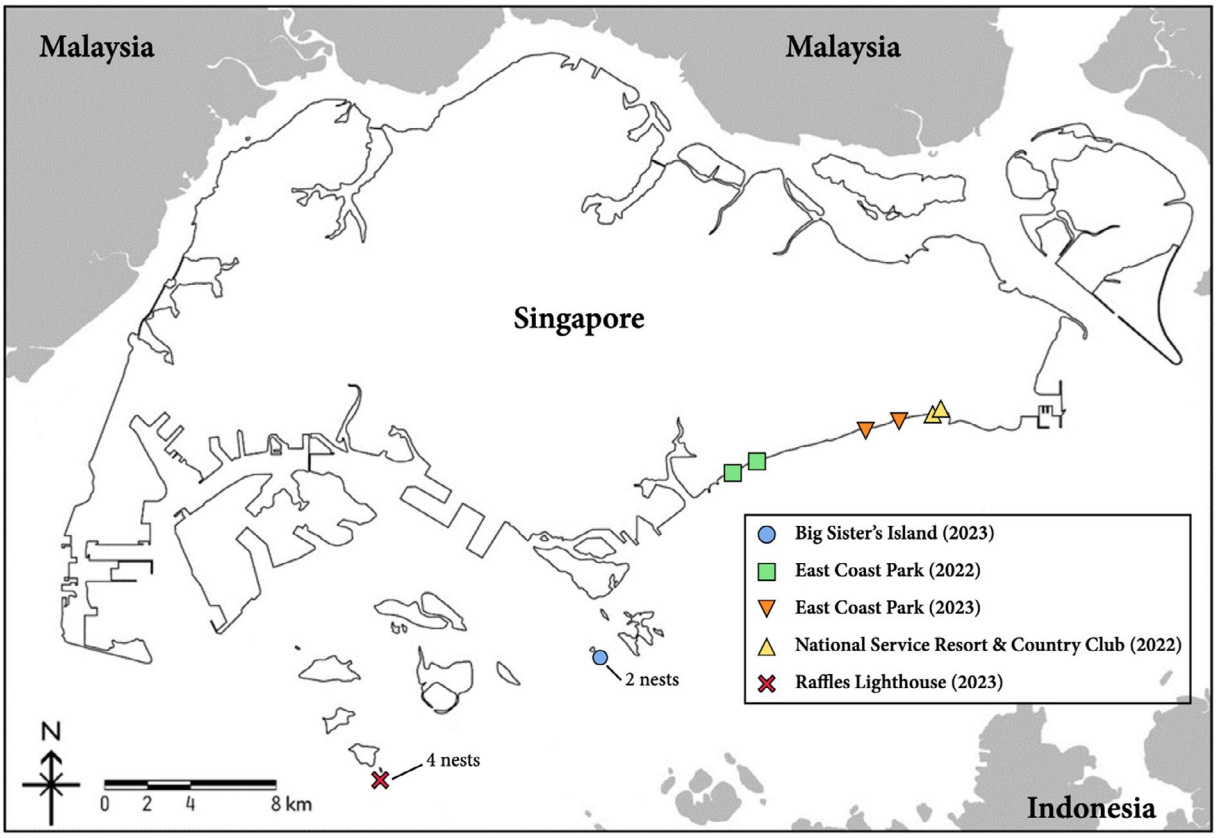

FIGURE 2 | Spatial distribution of hawksbill turtle nests sampled in Singapore during the 2022 and 2023 nesting seasons, limited to the southern coastlines where nesting naturally occurs (The map of Singapore is adapted from Tan and Low (2022)).

tubes, with three biological replicates per layer obtained by sampling different areas within the same depth. This yielded a total of 9 un-pooled samples per nest, with the exception of one nest where only three bottom-layer samples were obtained (Table S1). Dead hatchlings or unhatched eggs were stored in sterile specimen containers.

In order to obtain samples from live hatchlings, a modified sampling strategy was implemented during the 2023 nest visits. Namely, a wire mesh and a tile were laid across the nest to secure the opening, and sampling was carried out around Day 50 of the incubation period when most hatchlings had reached the nest's surface. As in 2022, sediment samples, dead hatchlings, and unhatched eggs were collected using the same methods, with the additional collection of negative field control sediment samples in 2023. These were collected from a single site 10 m upshore from each nest, dug to a depth of approximately 30 cm, and with three biological replicates in separate 15 mL falcon tubes.

In four out of the seven nests sampled in 2023, eye and oral swabs were collected from each live hatchling, using a separate swab per individual. The number of hatchlings swabbed per nest varied, ranging from 3 to 30, depending on nest size and hatchling survival (Table S1). To maintain the integrity of the samples, each swab was placed in a tube containing 3 mL of VTM and immediately kept on ice. After swabbing, the hatchlings from 3 of these 4 nests were placed in a tray for 15–20 min containing 100 mL of natural seawater collected on-site, which was later used for eDNA filtration. The tray was treated with 10,000 ppm sodium hypochlorite and rinsed with deionized water before use to prevent contamination. 100 mL of natural seawater and 100 mL of the water used to rinse the tray were collected as negative field controls.

All sediment samples, live hatchling swabs, dead hatchlings, and unhatched eggs were transported to CAVS on ice, where they were stored at −80°C until further processing. The eDNA water samples, contained in sterile falcon tubes, were also transported to the laboratory on ice. These samples were filtered as described in the following section, with the filters then stored at −80°C until further processing. Samples were processed and tested as a single batch once all nest collections for the year were completed. While all samples were ultimately preserved at −80°C, there were differences in storage durations before processing, with the oldest sediment and tissue samples being stored for up to 4 months, and the oldest swab and water filter samples being stored for up to 2 months. However, the samples underwent uniform handling when testing as a single batch, thus minimizing any potential bias in the results.

2.2 | Sample Processing

2.2.1 | Tissue and Swab Samples

Tumor and tissue samples from A6/8/20, A6/8/21, and the 2022 live green turtle case were subsampled and cut into smaller pieces before homogenization in Aquatic Animal Viral Transport Medium (AATM), which contains Minimal Essential Medium (MEM) supplemented with 5% fetal bovine serum,

3000 IU/mL penicillin G, 3 mg/mL streptomycin sulfate, 150 μg/mL kanamycin, and 37.5 IU/mL nystatin. For the hawksbill turtle nest-acquired samples, yolks were obtained from eggs and organs were obtained from the dead hatchlings and half-developed embryos. Similarly, 1 g of tissue from these samples was subsampled and homogenized.

All swabs preserved in VTM were vortexed for 15 s before decanting the liquid into 1.5 mL Eppendorf tubes. 200 μL was used for subsequent nucleic acid extraction. All homogenized tissue and decanted swab samples were digested in ATL buffer and proteinase K for 1 h before proceeding with DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen) in accordance with the manufacturer's instructions. Final elution was carried out in 100 μL of elution buffer.

2.2.2 | Environmental DNA Samples

Three types of eDNA samples were collected in this study, namely (i) tank seawater from the 2022 live green turtle case; (ii) natural seawater from the tray in which the hawksbill turtle hatchlings were placed prior to release; and (iii) sediment from hawksbill turtle nesting sites.

A total of 6 L of rehabilitation tank seawater was collected from the 2022 live green turtle case and transported to CAVS for immediate filtration. Of these, 4 L were collected before the turtle was reintroduced, and 2 L were collected 5 min after reintroduction. The "before" and "after" samples were treated separately and not pooled. Two filtration approaches were applied to the pre-reintroduction water to explore different eDNA capture strategies, given the limited volume of water available. 2 L were filtered using a vacuum pump through a 2.0 μm isopore membrane filter (Merck Millipore) to remove large particulates, followed by a 0.8 μm filter to capture the iron-virus flocculates. This was performed in accordance with the iron flocculation and vacuum pump filtration method as described in Ip et al. (2024). The other 2 L were divided into 1-L portions, each of which was filtered through a Sterivex filter (GP pressure filter unit, Merck Millipore) with a polyethersulfone (PES) membrane and a 0.22 μm pore size as described in Ip et al. (2024).

Due to logistical limitations, only 2 L of seawater were collected after the turtle was reintroduced into the tank. As a result, while two filtration methods were applied to the pre-reintroduction samples, only one method was used for the post-reintroduction sample. The post-reintroduction water sample was prioritized for iron flocculation and vacuum pump filtration, as DNA viral recovery rates from seawater have been shown to be higher with this method compared to the Sterivex filters (Ip et al. 2024).

Seawater samples of 100 mL each, collected from the tray where the hawksbill turtle hatchlings were placed prior to release, were taken from three nests in 2023. These samples, along with the negative natural seawater and deionized water controls (also 100 mL each), were subjected to iron flocculation and vacuum pump filtration as described above. All filters were stored at −80°C until nucleic acid extraction following the methodology outlined in Ip et al. (2024).

For the sediment eDNA samples, a spike-and-recovery experiment was conducted to test the feasibility of detecting ChHV5 from sediment using methods described by Farrell et al. (2022). A 1 g sample of flipper tumor tissue from the 2022 live green turtle case was homogenized in 3 mL of AATM. A 1:100 (10^{-2}) dilution of the tumor suspension in AATM was then prepared to simulate trace eDNA signals found in environmental samples. Subsequently, 50 μL of the 10^{-2} diluted tumor suspension was spiked into 10 g of negative sand sample, and 10 g of unspiked sand was also processed alongside the spiked sample for comparison. The sand used in this experiment was frozen without any buffer prior to spiking and thawed before use.

The spike-and-recovery experiment was run in duplicate. Using a 10 mL serological pipette, 20 mL of raw seawater from an unrelated site was added to each of the tubes containing either spiked or unspiked sand. The samples were shaken gently by hand and then set on a rotator-mixer for 1 h at room temperature with the following parameters: Orbital-50 rpm/01, Reciprocal-70/01, Vibro-0. Additional gentle shaking by hand was done every 15 min. After shaking, the samples were rested for 15 min to allow the sand to settle on the bottom of the tube. The supernatant was transferred to a 25 mL luer lock syringe using a 10 mL serological pipette, avoiding sediment transfer as much as possible. The spiked and unspiked samples were each filtered through a 0.22 μm Sterivex GP Pressure filter. The same sediment processing protocol was applied subsequently to all field samples, which were also frozen dry at −80°C and thawed before processing.

2.3 | Detection of ChHV5 DNA by Real-Time PCR

Real-time PCR was performed on all the samples collected from 2022 to 2023 in this study using the primers and probe described by Page-Karjian et al. (2015), which targets a conserved 173 bp fragment of the ChHV5 DNA polymerase (*UL30*) gene. The reactions were carried out in a total volume of 20 μL consisting of 10 μL of Maxima probe qPCR Master Mix (2×) (Thermo Fisher Scientific), 4 μL of nuclease-free water, 1 μL of a primers-probe mix (yielding final concentrations of 0.32 μM for each primer and probe), and 5 μL of template DNA. All reactions were performed on the CFX96 Real-time PCR System (Bio-Rad) or the 7900HT Fast Real-time PCR System (Applied Biosystems) with the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min. A valid amplification curve with a Ct value <40 is considered a positive detection.

A standard curve was generated using a synthesized plasmid containing the target region of *UL30*, which was serially diluted 10-fold from 10^8 copies to 1 copy per real-time PCR reaction. This curve was used for a preliminary assessment of assay efficiency and amplification reliability, providing an estimated detection threshold of 100 copies based on the presence of valid Ct values in three out of three technical replicates (Figure S2).

2.4 | DNA Sequencing

UL30 real-time PCR products from the A6/8/20 internal tumor, A6/8/21 internal tumor, A6/8/21 kidney, and the 2022 live

green turtle flipper tumor were sent for Sanger sequencing for sequence confirmation. The Sanger sequences were trimmed and assembled using Geneious v2023.1 (http://www.geneious.com; Kearse et al. 2012), and cross-referenced against the NCBI database using BLASTn (http://www.ncbi.nlm.nih.gov). These four samples were successfully sequenced, confirming the positive ChHV5 detections from the three green turtles. All *UL30* sequences generated have been deposited in GenBank under accession numbers PZ263070 to PZ263073.

Next-generation sequencing was conducted based on available technologies and methods at the point of testing. Tumor tissue samples from A6/8/20 and A6/8/21 were sequenced using shotgun-based high-throughput sequencing on the Illumina 151×151 bp iSeq 100 platform. The library preparation for A6/8/20 was performed using the Nextera Flex kit, while for A6/8/21, the Collibri ES DNA kit was used. The raw reads were adaptor-trimmed and aligned against the ChHV5 reference genome (GenBank accession number HQ878327.2; Ackermann et al. 2012) with bwa 0.7.17. The host mitochondrial reads from the same samples were similarly aligned against a complete green turtle mitochondrial reference genome (GenBank accession number NC_000886.1), with no filtering for nuclear mitochondrial pseudogenes (NUMTs).

Non-targeted shotgun-based Nanopore sequencing was performed on the 2022 live green turtle flipper and neck tumor tissues, as well as the water samples processed with both filtration methods, in order to leverage Nanopore's long-read capabilities. These sample extracts were end-repaired using the NEB Ultra II companion kit and MinION nanopore libraries were prepared using the Native Barcoding (EXP-NBD104) and ligation sequencing (SQK-LSK109) kits (Oxford Nanopore Technologies, Oxford, UK). Finalized DNA libraries of 14.04 ng were loaded on one reused Flo-MIN106 R9.4 flow cell (855 pores) and sequenced for up to 24 h. All runs were initiated with MinKNOW (22.12.7), with base calling using Guppy 6.4.6 accompanying adaptive sampling enrichment of the ChHV5 reference genome (Ackermann et al. 2012; GenBank accession no: HQ878327.2).

The raw fast5 sequence reads were exported and base-called using the High-Accuracy Model, trimmed by Guppy, and filtered to retain reads with a *q*-score greater than or equal to 8. The ChHV5 and host mitochondrial reads were then aligned to their respective reference genomes using Minimap2 v2.24 and assembled with medaka 1.7.2. The *q*-score ≥8 threshold was chosen to provide a balance between read quality and depth. At this threshold, the coverage was 99.86% with a mean depth of 18.93× for ChHV5 and 99.98% with a mean depth of 10.17× for the host mitochondrial genome. When applying a higher quality threshold (*q*-score ≥12), the coverage remained similar, but the mean depth decreased significantly to 9.39× for ChHV5 and 5.48× for the host mitochondrial genome.

2.5 | Viral Phylogenetics and Green Turtle Population Genetics

A total of 21 full ChHV5 genome sequences, including the reference genome HQ878327.2, were obtained from the study by Whitmore et al. (2021). To expand the dataset, an approximately 43 kbp partial ChHV5 genome (GenBank accession AY644454.1), covering genes *UL9* through *UL30*, was also obtained from the report by Herbst et al. (2004). Although incomplete, this partial genome still encompasses 20 genes and provides more genomic information compared to individual gene sequences, thus complementing the full genome dataset.

A multiple sequence alignment of the sequences was generated using MAFFT v. 7.490 (Katoh and Standley 2013) and trimmed to the 43 kbp region as described in Herbst et al. (2004) to ensure that only homologous regions were compared. Published green turtle mitochondrial control region (MTCR) sequences (Dethmers et al. 2006; Joseph et al. 2014, 2016) were also downloaded from GenBank and aligned with sequences generated from the three Singapore green turtle cases through high-throughput sequencing. RaxML-ng v1.1.0 was then used to infer a maximum-likelihood tree for each dataset using the GTR + I + G model of nucleotide substitution. Bootstrap support values were computed using 1000 replicates, and inferred trees were visualized using FigTree v1.4.4 (Rambaut 2018).

3 | Results

3.1 | Recovery of ChHV5 From Rehabilitation Tank Seawater and Green Turtle Tissue Samples

The real-time PCR results targeting a fragment of *UL30* revealed that ChHV5 could be readily detected in eDNA extracted from the rehabilitation tank water. Even with 2-L batches of seawater subsampled from a 3000-L tank, ChHV5 was readily detectable, with varying viral loads also potentially quantifiable depending on the presence/absence of the green turtle (Table S2). Specifically, we show that ChHV5 can persist in the water system, as the virus was still detectable 30 min after the turtle was removed from the tank (Ct = 31.29–31.49). This observation is consistent with the findings of Farrell et al. (2021), who also noted the potential persistence of ChHV5 in aquatic environments through their detection of ChHV5 DNA in a pond that was receiving outflow from turtle patient tanks. Nonetheless, further studies examining the half-life or decay of ChHV5 DNA in seawater are needed to understand the dynamics of ChHV5 virus persistence.

While the study by Ip et al. (2024) suggests that the iron flocculation and vacuum pump filtration method offers a higher virus recovery rate for DNA viruses compared to Sterivex filtration, the Ct values for ChHV5 detection were similar between filtration methods for the pre-reintroduction water samples despite differences in input volumes. The vacuum pump yielded a Ct value of 31.29 with 2 L of water processed, while the 2 samples filtered through the Sterivex syringe filters yielded Ct values of 31.45 and 31.49, each with 1 L of water processed (Table S2).

The ChHV5 detections in seawater were corroborated by positive ChHV5 detections in multiple tumor and organ samples from the 2022 live green turtle, with the flipper and neck tumor tissue samples showing the lowest Ct values of 19.52 and 17.67 respectively (Table S2). The Ct values for the swab samples (see Section 2.1.2) ranged from 25.44 to 37.07, indicating relatively lower viral loads compared to the direct tumor tissues. These results indicated that the virus can be

shed directly into the surrounding environment, with noticeable variability in viral shedding across the different organs swabbed. Furthermore, 5 min after the turtle was reintroduced into the tank, the Ct value of the water sample was observed to be 27.96, which suggests an increasing viral copy number in the tank water. Hence, the shedding of ChHV5 in the tank water was markedly responsive to the turtle's reintroduction, highlighting the potential for fine-scale monitoring of disease progression and recovery.

Long-read non-targeted shotgun sequencing was performed on the vacuum pump filtration samples from before and after turtle reintroduction, and the Sterivex filtration samples from before reintroduction. Enrichment was done bioinformatically during Nanopore sequencing by supplying the ChHV5 reference genome to MinKNOW for adaptive sampling. A total of four green turtle mitochondrial fragments (COI, COII, COIII, and ATP6) were recovered from the 6 L of rehabilitation tank water collectively. Interestingly, there were also six nanopore reads recovered that spanned a long region of ~15.5 kbp and that encompassed a portion of *UL30*. This is significant as it suggests that even without prior target enrichment, direct sequencing of eDNA samples may allow for simultaneous viral detection and population genetics analyses of host DNA, which has implications for integrative monitoring of viral prevalence and host population health.

3.2 | Phylogenetic Relationships of ChHV5 and Green Turtle Population Origins

A near-complete ChHV5 genome sequence of 132,563 bp from the union of viral sequences from the neck and flipper tumor tissue samples of the 2022 live green turtle case was assembled. The genome was determined by NCBI BLAST to be 99.48% identical to the ChHV5 reference genome (HQ878327.2). Only 20.5% and 8.43% of the ChHV5 genome could be recovered from the tumor tissues of A6/8/20 and A6/8/21 with Illumina sequencing respectively. These lower recovery rates likely reflect the carcasses being received in a state of decomposition, which resulted in DNA degradation. However, full green turtle mitogenomes of 16 kbp could be recovered from all three green turtle cases.

Phylogenetic analysis of a partial ~43 kbp region of the ChHV5 genome from the 2022 live green turtle tumor samples with 22 other publicly available genomes from Whitmore et al. (2021) and Herbst et al. (2004) highlights the need for more whole-genome sequencing of the virus from surveillance efforts in the region. The ChHV5 strain in Singapore's green turtle showed a paraphyletic relationship with the sequences obtained from Hawaii and the Gulf of Mexico (Figure 3). However, the resolution of these relationships is limited by the fact that most of the available genomes are from the USA and Hawaii. It is possible that the ChHV5 lineages in the Pacific could be more closely related to the populations from the Gulf of Mexico, potentially through the Panama Canal and/or the Drake Passage. However, further data about the current circulating strains in the region are required to explore this hypothesis.

For the turtle population genetic analysis, we extracted only the MTCR genes from the three green turtle mitogenomes and analyzed them alongside 23 other green turtle haplotypes following Joseph et al. (2014, 2016, 2023). The maximum likelihood tree showed that the Singapore green turtle cases were closely related to a cluster of haplotypes with members including C4, C5, C8, C14, CmP49, CmP87, and CmP89 (Figure S3). These haplotype members have been documented in foraging grounds in Brunei Bay and rookeries in East Malaysia such as Sarawak, Aru, and Berau Islands (Joseph et al. 2014, 2016, 2023).

3.3 | Feasibility of ChHV5 Detection From Sand and Active Biosurveillance From Nests

To evaluate the feasibility of detecting ChHV5 viral DNA from sand samples using our existing real-time PCR assay, clean sand samples were spiked with green turtle tumor suspensions from the 2022 live green turtle. The real-time PCR results of the spiked sand sample duplicates returned Ct values of 32.57 and 33.20, while the unspiked samples and negative controls returned no Ct value, demonstrating the feasibility of the real-time PCR assay for the detection of ChHV5 from sand eDNA samples (refer to Table S3). Although a direct comparison of Ct values between the sand and water samples is not possible in this study, a preliminary assessment of the existing assay's sensitivity suggests a conservative detection limit of approximately 100 copies per real-time PCR reaction. However, further virus recovery experiments will need to be performed using the correct sample matrix for assay validation, as ChHV5-positive material for spiking is currently limited.

Over the course of 2 years from sampling hawksbill turtle nests, we collected a total of 99 sand eDNA samples, 129 unhatched eggs and dead hatchlings, 11 seawater eDNA samples, and 62 live hatchling swabs (refer to Table S1). All hawksbill turtle nest-related samples tested negative for ChHV5 by real-time PCR. It is important to note that the negative eDNA results could reflect the detection limits of the assay, which may be influenced by factors such as sample volume, preservation methods, and assay sensitivity. Nonetheless, the combined results from all the sample types provide multiple lines of baseline evidence suggesting that local hawksbill turtles are currently free from the disease.

4 | Discussion

Although FP is recognized as a disease of concern to sea turtle populations worldwide, recent reviews have emphasized the lack of baseline data on its occurrence in local populations and the ongoing need for biosurveillance in order to assess changes in disease dynamics and potential population-level impacts (da Cruz et al. 2024; Whilde et al. 2024). In this study, we report the first molecular detections of ChHV5 in Singapore based on tumor tissues sampled over 3 years from two green turtle carcasses and one live green turtle. These cases reinforce the close association between ChHV5 and tumors in FP in a Southeast Asian context (Herbst 1994; Quackenbush et al. 2001; Jones et al. 2016). We also apply eDNA methods as non-invasive tools for the detection of ChHV5 viral DNA in seawater and sediment, highlighting their potential to support future disease monitoring and facilitate early disease detection.

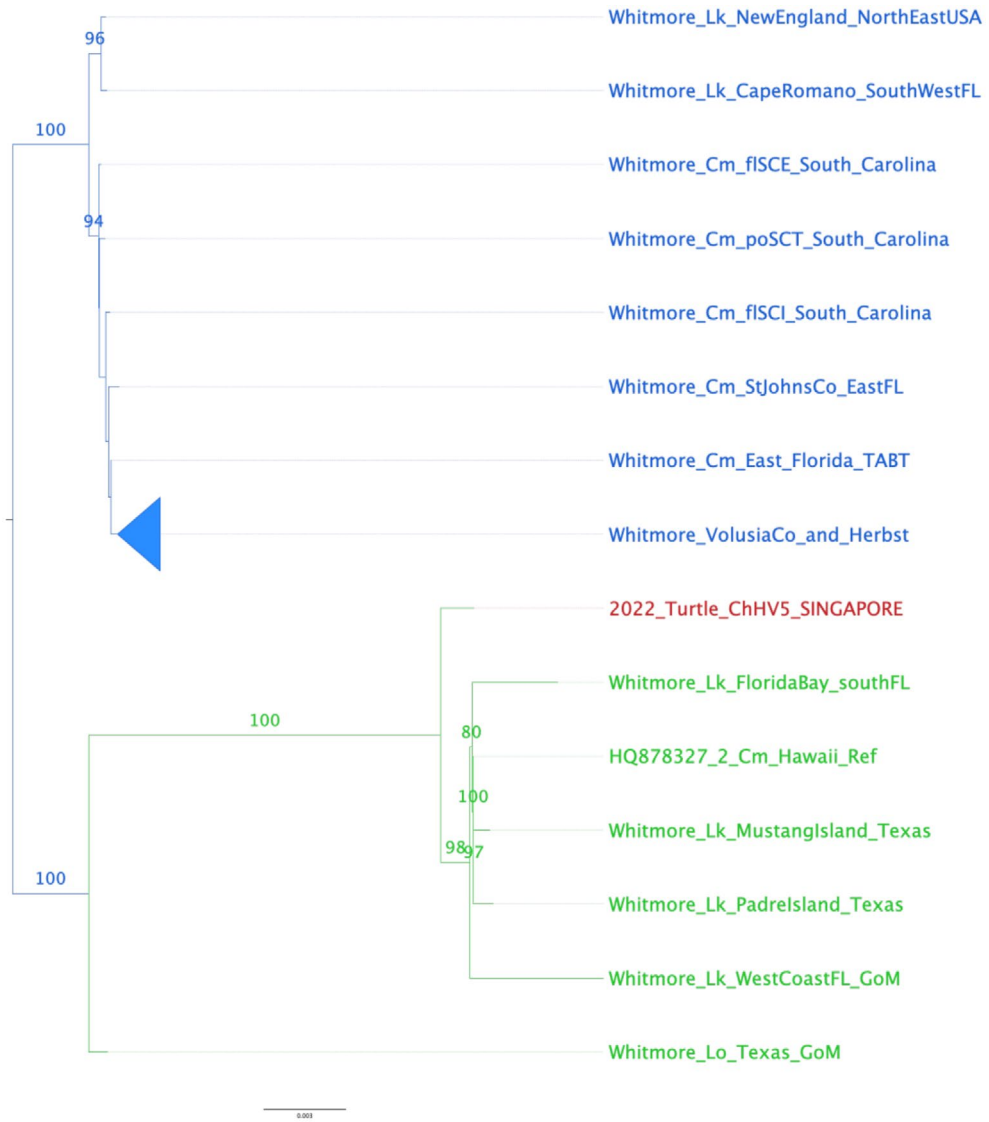

FIGURE 3 | Phylogenetic tree reconstruction from maximum likelihood analyses of a ~43 kbp partial region of the ChHV5 genome. The Singapore 2022 live green turtle case from our study is shown in red with 22 other publicly available ChHV5 sequences. To simplify the tree, the 9 sequences in the branch from Volusia County, Florida, and the sequence from Herbst et al. (2004) were collapsed. Sequences from the USA East Coast are shown in blue, while sequences from the Gulf of Mexico and Hawaii are shown in green.

Nanopore sequencing of the tumor samples from the 2022 live green turtle yielded the first near-complete ChHV5 genome from Southeast Asia, providing a key reference for a region that is currently under-represented in available ChHV5 datasets. Phylogenetic analyses comparing this genome with 22 other published genomes did not provide adequate resolution as most of the available data stem from around the USA East Coast, Gulf of Mexico, and Hawaii (Whitmore et al. 2021). It is possible that this limited geographic sampling may obscure the existence of distinct region-specific lineages within the Indo–Pacific. This result illustrates the need for increased sampling and deeper genomic characterization of ChHV5 strains in Southeast Asia, indicating that future efforts should prioritize this region to improve global coverage, potentially revealing novel evolutionary insights or previously uncharacterised lineages.

The mitochondrial genomes of the green turtles were also sequenced with non-targeted metagenomic sequencing on both the Illumina and Nanopore platforms to facilitate insights into the green turtle populations in Singapore. Focusing on the partial mitochondrial control region, we inferred that the local green turtle haplotypes were most closely related to those from foraging or nesting populations around Eastern Malaysia. From a management perspective, identifying the likely origins of these green turtles may provide context for tracing potential routes of ChHV5 exposure. This is particularly relevant because turtles undertake long-distance migrations and may encounter the virus at shared foraging grounds, where horizontal transmission is thought to occur (Li et al. 2017; Jones et al. 2016, 2020). Notably, analyses of mitochondrial haplotypes across East Asia have shown that green turtles from distant foraging grounds (e.g., South China and the Ryukyu Islands, Japan) often share natal origins in Southeast Asia, highlighting broad connectivity between foraging and nesting sites overall (Ng et al. 2024). Hence, future studies integrating the analysis of both host and viral genetic signals from the same samples are needed to

provide evidence for the establishment and strengthening of conservation networks among these connected habitats.

Given that reports of FP in sea turtles across Southeast Asia are relatively limited, our study explored the use of eDNA methods for the detection of ChHV5 viral DNA from water and sediment samples as a potential means to enhance disease biosurveillance efforts. Our eDNA-based detection of ChHV5 viral DNA in rehabilitation tank water demonstrates that viral shedding into the water column can be effectively tracked using real-time PCR (Farrell et al. 2021). In a captive setting, this approach enables non-invasive and rapid monitoring of disease progression, thereby enhancing our ability to address fundamental questions about the disease and design management policies such as quarantine, containment, and isolation. More recently, As-singkily et al. (2025) also demonstrated that eDNA extracted from seawater collected from natural shorelines in Indonesia can be used to recover haplotype information from nesting leatherback turtles (*Dermochelys coriacea*). Following this example, similar sampling approaches could potentially be adapted for monitoring ChHV5 in wild turtle populations, thus integrating disease surveillance with population genetic insights.

However, the application of eDNA methods in open marine environments faces more challenges due to the complex and dynamic nature of ocean currents, which can lead to stochasticity and dilution effects that may result in false negatives during detection (Sieber et al. 2020). Nonetheless, As-singkily et al. (2025) detected leatherback turtle DNA in seawater samples that were collected 12 h after the nesting females had left the beach, though calm waters, low waves, and weak currents may have contributed to the persistence of the signal. Additional factors such as the duration of host presence, the timing of pathogen release, and the vertical dispersion of the pathogen add further complexity (Miaud et al. 2019; Wittwer et al. 2018). Therefore, successful water eDNA field surveys require strategic timing and site selection, as well as the incorporation of biological and technical replicates with repeated sample collections over time (Hestetun et al. 2021; Mata et al. 2019).

In contrast to water sampling from marine environments, which is affected by ocean dynamics, sediment sampling has the potential to provide more localized eDNA signals, making it particularly useful in the field for site-specific monitoring. For example, Farrell et al. (2022) showed that ChHV5 viral DNA could be detected from the crawl sand left behind by a turtle hatchling as it crawled from its nest to the ocean. As such, we sought to validate the application of sediment eDNA sampling for ChHV5 detection using a spike-and-recovery experiment, eventually applying the method to sediment samples collected from hawksbill turtle nests in Singapore. The real-time PCR results from sediment, seawater, and tissue samples collected from the excavated nests provide baseline evidence that hawksbill nesting sites in Singapore currently show no ChHV5 presence. However, factors like high ambient temperatures and urban runoff may accelerate eDNA degradation and transport in sediment, and viral DNA could still be present at levels below the detection limits of our assay. Hence, future work will focus on refining our existing sediment eDNA method to better understand the sensitivity of our detection approach, exploring potential inhibition in different sample matrices, and increasing sampling efforts during future nesting seasons to determine the prevalence of ChHV5 in hawksbill turtles in Singapore.

While this study demonstrates the application of eDNA methods for preliminary health assessments in both green and hawksbill turtles in Singapore, we have identified several key priorities and knowledge gaps for future research on ChHV5 in the region. First, increased sampling to enable the co-analysis of host and viral genetic signals from matched samples or sites is a critical next step for linking viral occurrence with host population origins. Second, targeted screening of potential mechanical vectors, such as marine leeches (*Ozobranchus* spp.) that parasitise sea turtles and have been shown to carry viral loads, merits further study to clarify the mechanisms of ChHV5 transmission (Greenblatt et al. 2004; Farrell et al. 2021). Third, further refinement of eDNA methods (e.g., sediment and water), particularly when applied in natural settings, is key for supporting effective disease surveillance in wild turtle populations. Beyond eDNA and molecular detection tools, monitoring efforts in Singapore should also consider environmental factors such as urban runoff and contaminants, which may influence disease dynamics by altering immune responses and increasing host vulnerability to pathogens (Bosi and Desmarchelier 2018; Suzuki et al. 2020). Ultimately, integrative approaches that combine molecular, genomic, and ecological data should form the framework for future biosurveillance of ChHV5, supporting effective management strategies for emerging threats in endangered sea turtle populations.

5 | Conclusion

Our study reports the first near-complete ChHV5 genome from Southeast Asia, which serves as an important reference point for future phylogenetic and evolutionary studies and highlights the need for increased regional sampling. We demonstrated that viral shedding into rehabilitation tank water can be tracked non-invasively using eDNA sampling, illustrating the potential of this approach for broader pathogen surveillance. Lastly, the absence of detections of ChHV5 DNA in sediment taken from Hawksbill turtle nesting sites around Singapore provides baseline evidence that these sites are currently free from the presence of ChHV5. This underscores the importance of early detection in ongoing surveillance efforts for timely intervention in sea turtle populations. Overall, these methodologies and approaches establish a baseline for eDNA-based ChHV5 monitoring in Singapore and represent an important first step toward a regional framework for biosurveillance of sea turtles and their associated pathogens.

Author Contributions

The data analyses were performed by Y.C.A.I. and C.W.L. The manuscript draft and figures were prepared by Y.C.A.I., L.Y.T., C.W.L., and J.C., with inputs from X.H.T., C.H.E.L., and Z.Y.B.T. Post-mortems of the turtle cases were carried out by X.H.T. and C.H.E.L. The Illumina sequencing runs were carried out by Y.W. The eDNA sample and data collection was done by R.S.B., M.F.S., L.Y.T., and Y.C.A.I., with facilitation from C.H.Y.T. and J.Y. The study was supervised by K.B.H.E., S.F.C., C.J.F., and Z.Y.B.T. All authors contributed to the manuscript editing process, and approved the final version for publication.

Acknowledgments

We would like to thank Dr. Adrian Tan for his bioinformatic advice and pipelines. We would also like to thank Yap Hon Wah and Dr. Alfonso Lopez for rehabilitating the 2022 live green turtle, facilitating the collection of rehabilitation tank water and swab samples, and for providing the resected tumor tissue to the National Parks Board.

Funding

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All relevant data can be found within the manuscript and its Supporting Information. The assembled genome sequences and Sanger sequences are available on GenBank under the accession numbers PZ323048–PZ323050, PZ284982, and PZ263070–PZ263073.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Summary of the number of samples collected from 12 hawksbill turtle nests during 2022 and 2023. **Table S2:** ChHV5 real-time PCR detection results for samples taken from the 2022 live green turtle case. **Table S3:** ChHV5 real-time PCR detection results of sand samples spiked with 10^{-2} diluted tumor suspension from the 2022 live green turtle case. **Figure S1:** Field photograph of the live green turtle in this study (2022), exhibiting tumors on the neck and left fore-flipper consistent with fibropapillomatosis. **Figure S2:** A standard curve for the ChHV5 real-time PCR assay was generated by plotting triplicate Ct values against the $\log_{10}$-transformed

plasmid copy numbers, which ranged from 10^0 to 10^8 copies. The slope, *y*-intercept, correlation coefficient (R^2), and reaction efficiency are indicated on the plot. The estimated detection threshold for the ChHV5 assay was determined to be 100 (10^2) copies based on the presence of valid Ct values in three out of three technical replicates. **Figure S3:** Phylogenetic tree reconstruction from maximum likelihood analyses of the three green turtle mitochondrial control region sequences generated from our study (highlighted in red), with publicly available green turtle mitochondrial control region sequences. The sequences in blue represent Clade I according to Dethmers et al. (2006).