CASE REPORT

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First report of fibropapillomatosis (FP) and *Chelonid alphaherpesvirus* 5 (ChHV5) in a green sea turtle (*Chelonia mydas*) from the historically fibropapillomatosis-free Fernando de Noronha Archipelago, Northeastern Brazil

Primeiro caso de fibropapilomatose (FP) e de Chelonid alphaherpesvirus 5 (ChHV5) em tartaruga-verde (Chelonia mydas) no Arquipélago de Fernando de Noronha, Nordeste do Brasil

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ABSTRACT

Fibropapillomatosis (FP) is an infectious disease caused by *Chelonid alphaherpesvirus* 5 (ChHV5). Nevertheless, its clinical manifestations are considered multifactorial. Due to its relevance, FP is currently monitored in sea turtle populations in the United States, Australia, Caribbean, and Brazil. Between 2000 and 2020, the TAMAR Project/ TAMAR Project Foundation analyzed the prevalence of FP in nine states and oceanic islands along the Brazilian coast, including Fernando de Noronha Archipelago (FNA), a historically FP-free area. A total of 4,435 green sea turtles (*Chelonia mydas*) were monitored from 2010 to 2016. Additionally, in 2012 and 2014, 43 FP-free skin samples were analyzed for ChHV5 using a qualitative PCR for the UL30 polymerase (pol) sequence. In 2015, a bilateral ocular nodule characterized as an FP tumor was reported in one of the monitored individuals undergoing rehabilitation. Tissue samples were collected following surgical removal of the tumor. Characterization of a 454 bp UL30 polymerase gene revealed a ChHV5 sequence previously reported in other areas of the Atlantic Brazilian coast. In the years following this finding from January 2017 to March 2020, a total of 360 *C. mydas* were monitored in the same area and no FP tumors were detected. This is the first report of FP and the first detection of ChHV5 in FNA, a finding of great concern considering this site's historical absence of FP occurrence. This study highlights the importance of monitoring this disease in historically FP-free areas of the Brazilian Atlantic coast.

Keywords: *Alphaherpesvirus*. Fibropapilloma. Brazilian Atlantic coast. Fernando de Noronha Archipelago. Marine turtles. Tumors.

RESUMO

A fibropapilomatose (FP) é uma doença infecciosa causada pelo *Chelonid alphaherpesvirus* 5 (ChHV5). No entanto, as manifestações clínicas da doença são consideradas multifatoriais. Esta doença é monitorada atualmente em populações de tartarugas marinhas nos EUA, Austrália, Caribe e Brasil. Desde 2000, o Projeto TAMAR/Fundação Projeto TAMAR

analisa a presença de FP em nove estados da costa brasileira e ilhas oceânicas, incluindo o arquipélago de Fernando de Noronha, uma área historicamente livre de FP. Um total de 4.435 indivíduos de *Chelonia mydas* foram monitorados de 2010 a 2016 e 43 amostras de pele foram analisadas para detectar ChHV5 em 2012 e 2014 com o objetivo de avaliar a presença do vírus em tecidos sem FP, usando uma PCR qualitativa para detecção de sequências do gene da UL30 polimerase. Em 2015, uma tartaruga verde (*C. mydas*) foi relatada com um nódulo ocular bilateral caracterizado como FP. Amostras de tecido foram coletadas durante sua reabilitação e procedimento cirúrgico para remover o tumor. A caracterização parcial de uma sequência de 454 bp do gene UL30 polimerase detectou ChHV5 anteriormente relatado em outras áreas da costa atlântica brasileira. Após estes achados, de janeiro de 2017 a março de 2020, um total de 360 indivíduos de *C. mydas* foram monitorados e nenhum caso de FP foi registrado. Este é o primeiro relato de FP e a primeira caracterização de ChHV5 no arquipélago de Fernando de Noronha, uma questão preocupante e que ressalta a importância do monitoramento desta doença em áreas historicamente livres de FP na costa atlântica brasileira.

Palavras-chave: *Alphaherpesvirus.* Fibropapiloma. Costa atlântica brasileira. Arquipélago de Fernando de Noronha. Tartarugas marinhas. Tumores.

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Fibropapillomatosis (FP) is a debilitating and multifactorial neoplastic disease associated with *Chelonid alphaherpesvirus* 5 (ChHV5) infection (Quackenbush et al., 1998; Lackovich et al., 1999). After its first report in the New York Aquarium in 1938 (Smith & Coates, 1938), FP has been detected and monitored in several regions of the world: the Americas (United States - Florida and Hawaii), Africa (Príncipe Island, Guinea-Bissau, and Mauritania), Australia (Queensland), and Brazil (Foley et al., 2005; Baptistotte, 2016; Jones et al., 2020). FP affects all sea turtle species and has been observed in all oceans (Herbst, 1994; Alfaro-Núñez et al., 2014), with the highest prevalence in green sea turtles (*Chelonia mydas*) (Baptistotte, 2016). ChHV5 is a double-stranded DNA virus (*Alphaherpesvirinae* subfamily, *Scutavirus* genus) with a genome size of approximately 132 kb (Ackermann et al., 2012). Its characteristics have been recently elucidated due to the expansion of molecular detection methods and studies on several regions of the viral genome and phylogenetic analyzes (Ackermann et al., 2012; Jones et al., 2020). ChHV5 has a short replication cycle, the ability to destroy infected cells and establish latent infection, and amino acid changes in the viral capsid. Nevertheless, despite such characteristics, FP may be related to its dispersal, particularly in sea turtle feeding areas, where the congregation of juvenile turtles may promote a greater infection rate or super-spreader individuals (Alfaro-Núñez et al., 2014; Work et al., 2015). However, vectors (i.e., marine leeches), ChHV5 environmental persistence, and potential horizontal transmission through secretions should also be considered (Greenblatt et al., 2004; Monezi et al., 2016; Chaves et al., 2017). The prevalence and severity of the lesions may also be associated with environmental factors, such as water quality (Santos et al., 2010), temperature (Herbst et al., 1995), presence of biotoxins (Landsberg et al., 1990), land use (Van Houtan et al., 2010), pollutants (Silva et al., 2016), and geographic factors (Rossi et al., 2016). Due to the wide range of factors that can lead to FP development, long-term monitoring studies are fundamental to understanding the relationship between environmental changes, virus evolution, infection rates, and the prevalence of the disease in distinct areas of the globe. This study presents 20-year FP monitoring data (2000 to 2020) from Fernando de Noronha Archipelago (FNA), Brazil, the results of detection of ChHV5 in skin samples, performed in 2012 and 2014, whose aim was the analysis of the virus presence in non-tumor tissues, and the first report and histological characterization of FP with molecular analysis of partial 454 bp UL30 polymerase gene of ChHV5 in *C. mydas* in the region.

FNA is an offshore volcanic archipelago located 545 km off the coast of Pernambuco State, northeastern Brazil (51' 13" N, 32º 25' 25" O) (Figure 1). It consists of one main island and approximately 18 small islets, encompassing a total land area of 26 km^2 (Garla et al., 2006). Aiming for

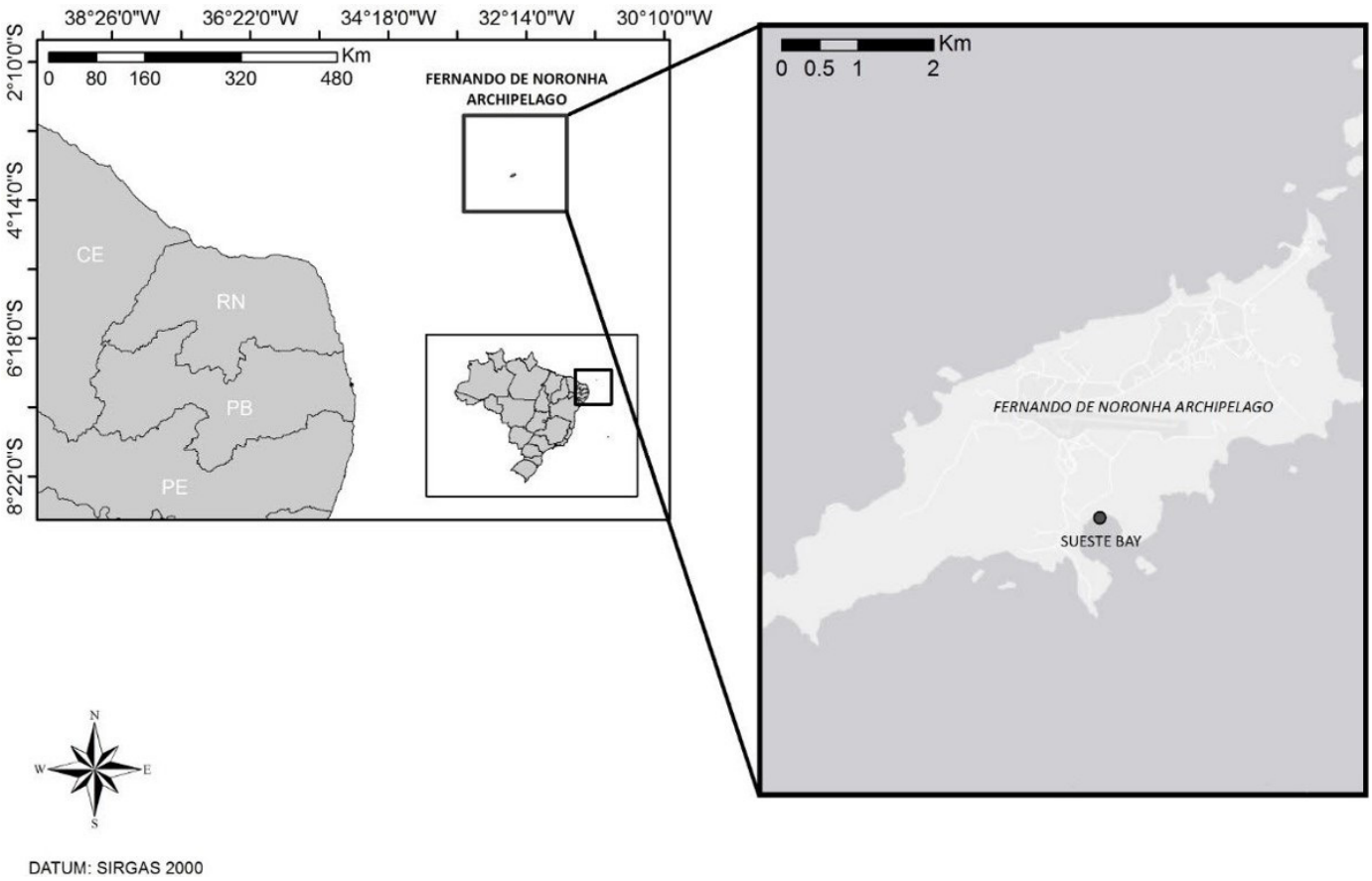

Figure 1 – Fernando de Noronha Archipelago/Pernambuco, Brazil. Capture site (Sueste Bay) of the green sea turtle with FP. Source: Projeto Cetáceos da Costa Branca - Universidade do Estado do Rio Grande do Norte (PCCB-UERN). Edited by Giovanna Almeida Santoro.

sea turtle conservation, the Fundação Projeto TAMAR (TAMAR Project Foundation) has been permanently stationed in FNA, as well as in selected sites along the Brazil coast since 1984 (Marcovaldi & Marcovaldi, 1999). Most specifically, the TAMAR Project has been monitoring FP in all sea turtle species occurring in FNA since 2000. Between 2000 and 2016, the resulting monitoring data was stored at the Sistema de Informação sobre Tartarugas Marinhas (SITAMAR – Sea Turtle Information System) data bank, developed and maintained by the Fundação Projeto TAMAR (TAMAR Project Foundation). During that period, according to SITAMAR, 4,435 *Chelonia mydas* specimens were captured. All procedures were performed in full compliance with specific federal permits issued by the Brazilian Ministry of Environment and approved by the Biodiversity Information and Authorization System (SISBIO 32636-9).

All specimens collected were tagged and their biometric data recorded. During this monitoring period, two skin collections were performed in a total of 43 individuals (22 in 2012 and 21 in 2014) without FP tumors, and with curvilinear carapace length (CCL) ranging between 30 and 70 centimeters. Our goal was to evaluate the occurrence of ChHV5 in non-tumor-bearing individuals, based on the hypothesis of latent ChHV5 persistence in sea turtle tissues, previously reported in five different species worldwide (Alfaro-Nuñez et al., 2014). The first FP case was observed in a *C. mydas* specimen (CCL=84 centimeters) captured by the TAMAR Project at Sueste Bay, on December 28, 2015 (Figure 1), and translocated by airplane to Natal (Rio Grande do Norte state) on January 7, 2016, for rehabilitation and surgical procedures. The ocular tumors were characterized as FP, presenting epidermal and hyperplastic stromal proliferation, epithelial cells with cytoplasmic vacuolization, epidermal cell degeneration, and fibroblast proliferation (Figure 2).

The second FP case was in a subadult *C. mydas* specimen (CCL=87.5 cm), captured on October 22, 2016. This individual was first captured in 2005, with 41 cm CCL and no FP cutaneous tumors upon physical inspection. Nevertheless, the nodule was not removed and this second individual was returned to the wild (Santos, personal communication).

The skin samples from all 43 individuals captured in 2012 and 2014, as well as the ocular tumor samples, were stored in 100% ethanol and preserved at -20ºC until processed (Krafft et al., 2005). Total genomic DNA was extracted one

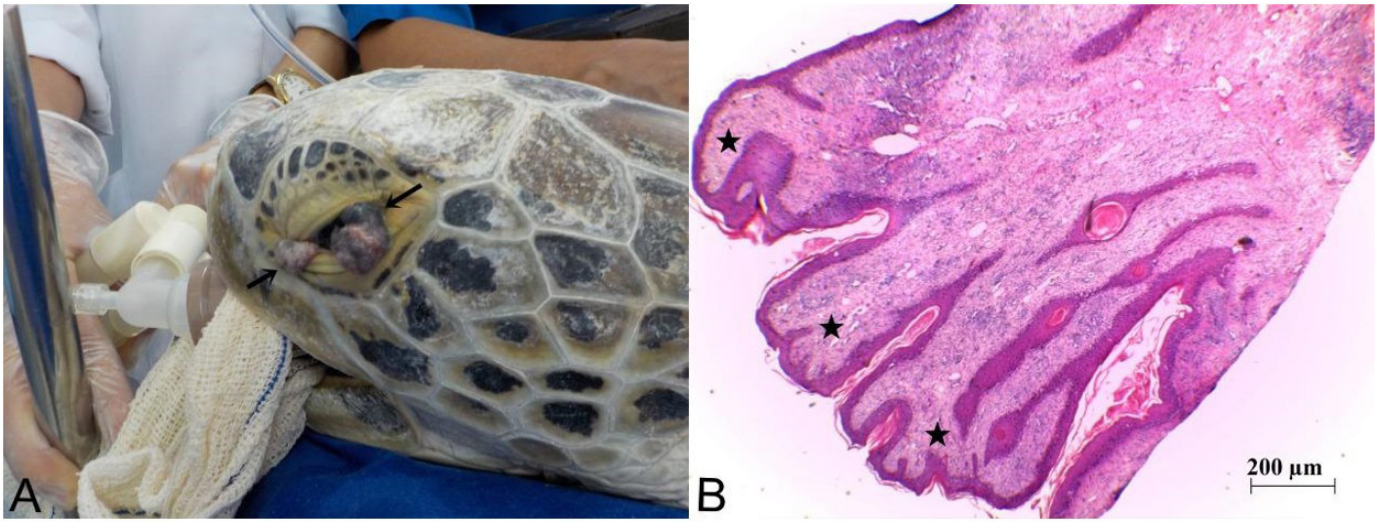

Figure 2 – Ocular fibropapillomas found on the examined green sea turtle (arrows); (B) Histopathological section of fibropapillomas: Note the papillary projections (stars).

month after sample collection using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The purified DNA was used for partial UL30-pol gene detection (Quackenbush et al., 2001), while UL18 and UL27 partial gene sequences were used only to detect ChHV5 in tumor samples (Alfaro-Nuñez & Gilbert, 2014). Nuclease-free water was used as a negative control and a previously detected ChHV5-positive DNA sample was used as a positive control. A PCR assay using 2.5 µL of DNA extract in a 25 µL reaction with Platinum Taq polymerase (Life Technologies, USA) was used to detect the UL30-pol, UL18, and UL27 partial gene sequences. Each reaction contained 1 × PCR buffer, 1 mM $MgSO_4$, 0.2 mM each primer, 0.2 mM dNTP mixture and 0.2 µL polymerase. The PCR cycles to detect UL30-pol, and UL18 and UL27 genes were performed according to Rodenbusch et al. (2014) and Alfaro-Nuñez & Gilbert (2014), respectively. Each PCR product was visualized in a 2% agarose gel. The detected amplified products were purified using the GTX PCR DNA and Gel Band Purification kit (GE Healthcare, USA), following the manufacturer's instructions. Amplicons were sequenced using BigDye Terminator v 3.1 (Applied Biosystems, USA) and ABI Prism 310 Genetics (Applied Biosystems, USA). Sequences were aligned using Clustal W, and the neighbor-joining method was utilized to perform the phylogenetic tree, according to the Kimura 2-parameter model. Bootstrap values were calculated out of 1,000 replicates. All the analyses were carried out using MEGA-X. An unrooted nucleotide phylogenetic tree based on the partial (454 bp) DNA polymerase gene of ChHV5 was generated.

The presence of ChHV5 was not detected in skin samples, only in the ocular tumor (UL30-pol, UL18, and UL27). A 457 bp UL30 nucleotide gene sequence was submitted to GenBank under access number MH101748, and the phylogenetic analyses were conducted with 454 bp UL30 polymerase gene to analyze a larger number of deposited sequences. The partial UL30 polymerase gene sequence revealed that the herpesvirus detected herein clustered with Atlantic ChHV5 associated with previous sequences detected in the skin and FP samples of green sea turtles in southeastern Brazil (Rodenbusch et al., 2014; Domiciano et al., 2019), and the leatherback sea turtle, a pelagic sea turtle species, in northeastern Brazil. Our partial 454 bp UL30 sequence gene presented 100% identity with sequences detected in FP of green sea turtle (São Paulo, Brazil) (JN938585) and pulmonary fibromyxoid sarcoma of leatherback sea turtle (Sergipe, Brazil) (MK357710), from 2011 and 2019, respectively. Higher identity was identified with other ChHV5 variants detected in Paraná and São Paulo, in Brazil (99.7%, MH144348, and MH101744, respectively), Puerto Rico (99.7%, JN580279), Espírito Santo (Brazil) (99.5%, JN938586), and Costa Rica Pacific coast (99.5%, KP724836). Phylogenetic analyses of UL30 showed that the alphaherpesvirus detected in our study clustered with the Atlantic ChHV5 group identified in samples from 2012 and later, according to GenBank and previous studies (Figure 3) (Patricio et al., 2012; Rodenbusch et al., 2014; Domiciano et al., 2019). The partial UL30 polymerase gene sequence also clustered with a sequence detected in pulmonary lesions of a leatherback sea turtle, emphasizing the importance of understanding ChHV5 transmission in other tissue lesions and the pelagic environment (Díaz-Delgado et al., 2019) (Figure 3).

This study describes the first histological characterization of fibropapillomatosis in green sea turtles in the Fernando

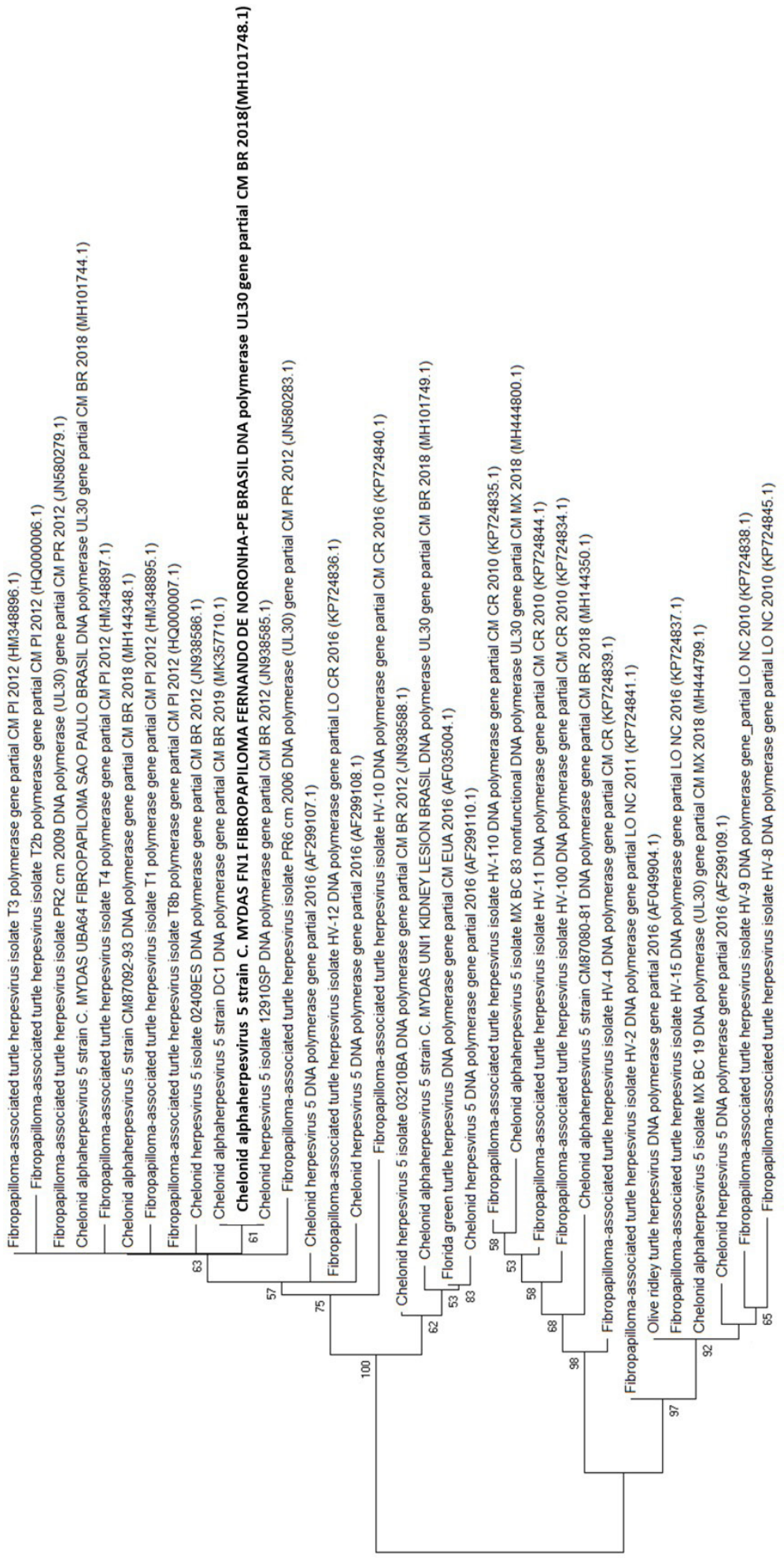

Figure 3 – Unrooted nucleotide phylogenetic tree based on partial (454 bp) DNA polymerase gene of ChHV5 using the neighbor-joining distances method and Kimura two-parameter distance correction model. Bootstrap values (1,000 pseudoreplicates) of >50% are indicated at the branch nodes. The scale bar represents the number of nucleotide substitutions per site. The ChHV5 nucleotide sequence detected in this study is in bold, with GenBank accession numbers in parenthesis. BR: Brazil; CR: Costa Rica; HI: Hawaii; NC: Nicaragua; PI: Principe Island – West Africa; PR: Puerto Rico. Sea turtle species: CM: *Chelonia mydas*; DC: *Dermochelys coriacea*; LO: *Lepidochelys olivacea*.

de Noronha archipelago, northeastern Brazil. Additionally, this is the first molecular detection of ChHV5 in this area, where fibropapillomatosis had not been reported until 2015. The presence of ChHV5 in this species, previously reported in continental areas of Paraná (south), São Paulo, Rio de Janeiro, and Espírito Santo (southeastern), Ceará, and the Rio Grande do Norte (northeastern) states suggests that the virus is widely distributed along the Brazilian Atlantic coast, including insular environments (Rossi et al., 2021; Rodenbusch et al., 2014).

It is still unknown if the presence of ChHV5 and fibropapillomatosis may influence the health of the specimens comprising the archipelago's mixed stock and population of green sea turtles. Since our findings in 2015, a total of 360 green sea turtle individuals were monitored between January 2017 and March 2020, without a single detection of fibropapilloma tumors. Since the archipelago is a national protected area, it is potentially under lower anthropic impact than other continental areas of Brazil. Because fibropapillomatosis was detected in consecutive years (2015 and 2016), studying focal points of ChHV5 and fibropapilloma occurrence highlight the importance of continuously monitoring this disease in the Fernando de Noronha archipelago, a key repository of green sea turtles in the Brazilian Atlantic coast and feeding area in the southern Atlantic.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Statement

The article was developed from data concerning rehabilitation of sea turtle, rescued by a Beach Monitoring Project (PMP-Potiguar Basin) that was licenses from the Federal Inspection Agency (IBAMA) - SISBIO 32636-9 – for the execution of this activity. All surgery and rehabilitation procedures were developed considering the protocols in force in the literature in the area.

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