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First Olive Ridley Sea Turtle (*Lepidochelys olivacea*) Stranding in Texas, USA, and Identification of a Chelonid AlphaHerpesvirus 5 (ChHV5) Variant Present in Tumor Tissue

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The Olive Ridley Sea Turtle (*Lepidochelys olivacea*), listed in the IUCN Red List as Vulnerable (Abreu-Grobois and Plotkin 2008), is considered to be the most globally abundant sea turtle species (Spotila 2004; Abreu-Grobois and Plotkin 2008; Valverde et al. 2012) but also the least abundant sea turtle species in the western Atlantic region (Marcovaldi 2001). Olive Ridley Sea Turtles do not nest in the continental USA but are observed in U.S. waters off the coasts of California, Hawaii, and southern Florida (Balazs 1985; Foley et al. 2003; NMFS and USFWS 2014). Rare sightings and captures of Olive Ridley Sea Turtles have been reported outside of their typical distribution range in the greater Caribbean near Puerto Rico, the Dominican Republic, Cuba, and Trinidad (Aguayo 1953; Carr 1957; Caldwell and Erdman 1969; Varona 1974; Carr et al. 1982; Reichart 1993; Horta et al. 2000; Moncada-G. et al. 2000) and in waters as far north as the Grand Banks Region near Canada (Stokes and Epperly 2006). The range of the Olive Ridley Sea Turtle is not known to extend into the Gulf of Mexico (GoM; NMFS and USFWS 2014), and geographic overlap with Kemp's Ridley Sea Turtles (*Lepidochelys kempii*) in the western Atlantic region has been documented only near south Florida (Foley et al. 2003).

Fibropapillomatosis (FP), a disease characterized by often debilitating internal and external tumors (Herbst 1994), was first reported in Florida in the 1930s (Lucke 1938; Smith and Coates 1938) with anecdotal reports as early as the late 1800's (Cruz 1985). Fibropapillomatosis affects Green Sea Turtles (*Chelonia mydas*) globally (Aguirre et al. 1999; Alfaro-Núñez et al. 2014; Seminoff et al. 2015). Of the seven sea turtle species, FP primarily affects Green Sea Turtles (Williams et al. 1994) and has been documented in every major ocean basin that hosts them (Herbst

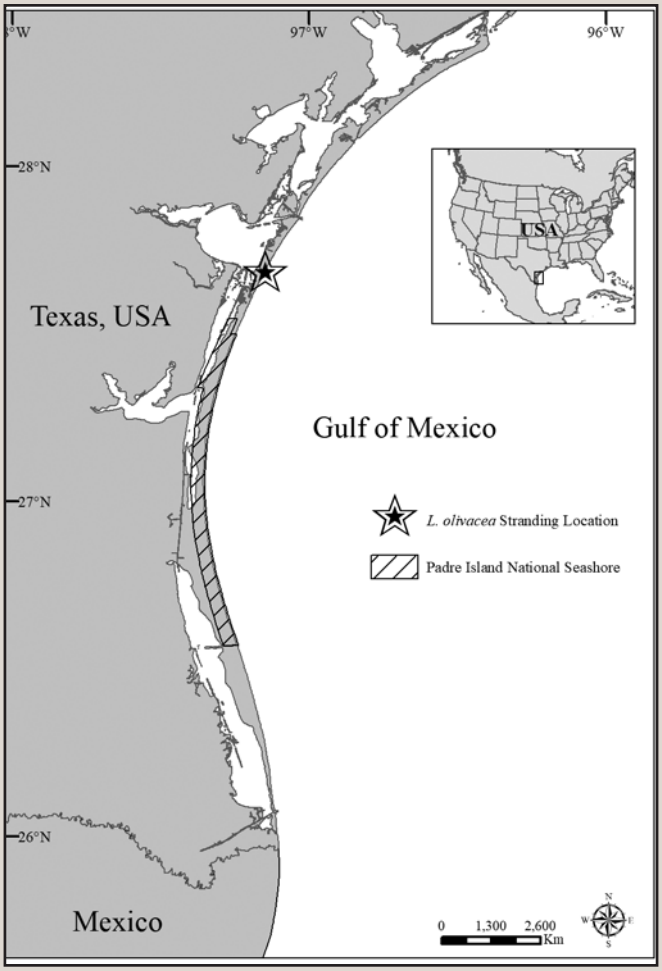

FIG. 1. Location of the Olive Ridley Sea Turtle (*Lepidochelys olivacea*) stranding on the south Texas coast, USA.

FIG. 2. Stranded Olive Ridley Sea Turtle (*Lepidochelys olivacea*) carcass recovered at Mustang Island State Park, Mustang Island, Texas, USA and necropsied at Padre Island National Seashore, Texas, USA. Photo provided courtesy of the National Park Service.

1994). However, FP tumors have been reported in all other sea turtle species worldwide, including Flatback (*Natator depressus*), Hawksbill (*Eretmochelys imbricata*), Kemp's Ridley, Leatherback (*Dermochelys coriacea*), Loggerhead (*Caretta caretta*), Olive Ridley, and hybrids of these species (Harshbarger 1991; Barragan and Sarti 1994; Herbst 1994; Limpus and Miller 1994; Aguirre et al. 1999; D'Amato and Moraes-Neto 2000; Huerta et al. 2002; Herbst et al. 2004; Williams et al. 2006; Jones et al. 2020).

FP was first observed in Olive Ridley Sea Turtles in Costa Rica in 1982 at Ostional National Wildlife Refuge (NWR; Cornelius and Robinson 1983; Limpus and Miller 1994) but was not confirmed via histopathology until 1999 (Aguirre et al. 1999). Chaves et al. (2013) subsequently detected chelonid alphaherpesvirus 5 (ChHV5) in diseased and healthy tissues from Olive Ridley Sea Turtles at Ostional NWR. Fibropapilloma tumors have been observed in Olive Ridley Sea Turtles nesting on the Pacific coasts of Costa Rica (Herbst 1994; Aguirre et al. 1999) and Nicaragua (Chaves et al. 2017), nesting in India (NMFS and USFWS 2014), foraging off the Pacific coast of Mexico (Mejía-Radillo et al. 2019), and stranded in Chile (Álvarez-Varas et al. 2019).

FP is a transmissible disease associated with infection by ChHV5 (Herbst 1994; Quackenbush et al. 2001; Alfaro-Núñez et al. 2014; Chaves et al. 2017). Environmental pollution and anthropogenic habitat degradation are proposed disease cofactors, but the etiopathogenesis of the disease has not been fully elucidated (Foley et al. 2005; Van Houtan et al. 2010). ChHV5 has been detected in the tumor tissue of turtles with FP (Quackenbush et al. 1998, 2001; Lu et al. 2000; Page-Karjian et al. 2012), but also in the tissue of healthy turtles, which may indicate latent or early infection (Page-Karjian et al. 2012, 2020; Alfaro-Núñez et al. 2014). Several regional variants of ChHV5 have been identified (Ene et al. 2005; Alfaro-Núñez et al. 2014)

Herein, we report the first confirmed occurrence of an Olive Ridley Sea Turtle stranding on the western GoM coast in Texas, USA (Fig. 1) since the establishment of the Texas Sea Turtle Stranding and Salvage Network (STSSN) in 1980. Additionally, we identify the ChHV5 viral sequences specific to the DNA polymerase gene (UL30) extracted from tissue collected from a tumor present on the stranded Olive Ridley Sea Turtle and investigate their relatedness to ChHV5 sequences documented in Green, Loggerhead, and Olive Ridley Sea Turtles sampled at other locations.

MATERIALS AND METHODS

Stranded Sea Turtle Documentation, Salvage, and Recovery.—From 1980–2020, live and dead sea turtles stranded on the Texas, USA coast were located and documented by the Texas STSSN via reports made by the public and during systematic patrols for stranded and nesting Kemp's Ridley Sea Turtles. The ability of the Texas STSSN to locate and respond to stranded sea turtles varied geographically and temporally. The most consistent documentation of stranded sea turtles occurred between April and mid-July of each year, during the patrols for nesting Kemp's Ridley Sea Turtles, wherein most of the Texas GoM coast was traversed repeatedly each day by trained sea turtle patrollers (Shaver et al. 2016). Stranded turtles were identified to species, photographed, and visually examined for external tumors associated with FP. Live stranded turtles were recovered and transported to rehabilitation facilities in close proximity to the stranding location. Dead stranded turtles were either salvaged for necropsy or buried at their stranding location, contingent on the species, level of decomposition, and remoteness of the stranding location. Sea turtle carcasses salvaged from stranding locations along the south Texas coast (Mustang Island to Boca

PHOTO BY BAS

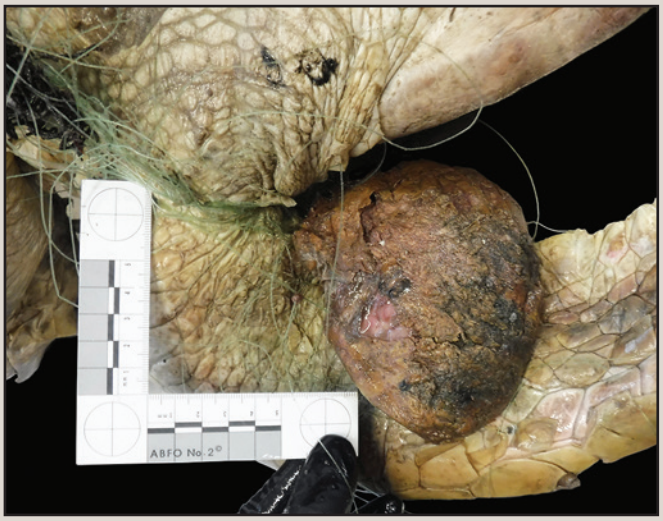

FIG. 3. Pedunculated, ulcerated tumor (consistent with a large fibropapilloma) on the ventral aspect of front right flipper of the Olive Ridley Sea Turtle (*Lepidochelys olivacea*) necropsied at Padre Island National Seashore, Texas, USA. The base of the tumor is entangled in monofilament line. Photo provided courtesy of the National Park Service.

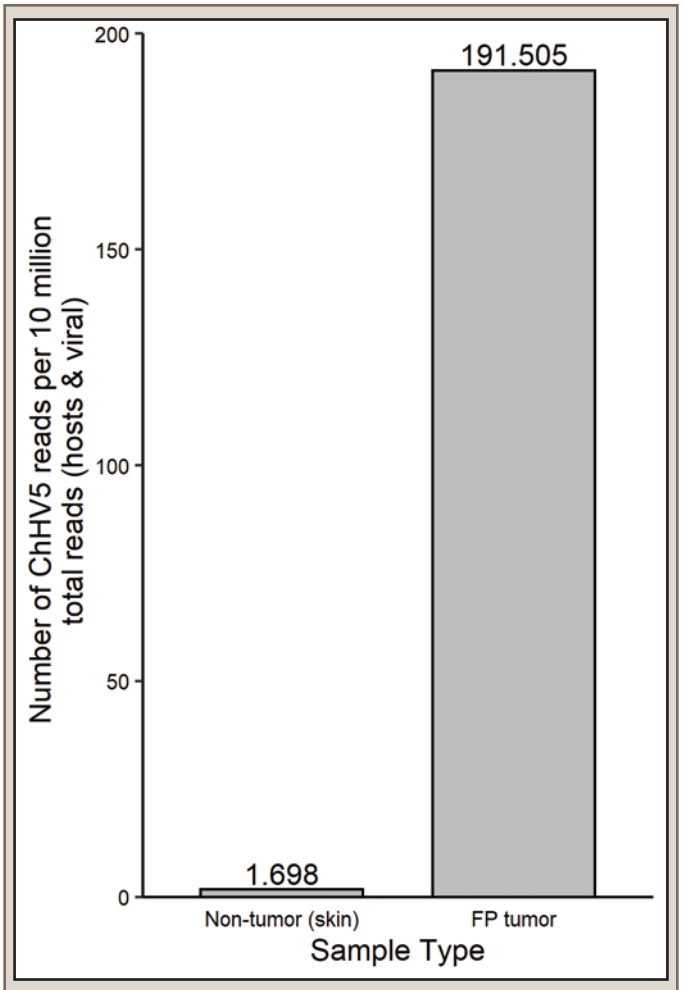

FIG. 4. ChHV5 viral load detected in the non-tumor and the fibropapilloma tumor sample from the stranded Olive Ridley Sea Turtle (*Lepidochelys olivacea*) carcass. Viral DNA was quantified by Illumina sequencing.

Chica Beach) were stored frozen until necropsy, mostly at Padre Island National Seashore (PAIS), Nueces County, Texas.

On 27 July 2019, a dead stranded sea turtle was salvaged from Mustang Island State Park, Mustang Island, Texas (27.66901°N, 97.17181°W; WGS 84; Fig. 1). The turtle was documented on the standardized STSSN form and measured for maximum straight-line carapace length (SCL_{max}; from the nuchal notch to the posterior tip), minimum straight-line carapace length (SCL_{min}; from the nuchal notch to the posterior notch), and straight-line carapace width (SCW; widest point). External morphology was examined to identify species. The carcass was frozen and later thawed for the necropsy conducted at PAIS on 20 August 2019 (Fig. 2). During necropsy, further attempts were made to confirm species by collecting samples of skin tissue using 6-mm biopsy punches (Integra Miltex, York, Pennsylvania, USA), which were stored in ethanol at room temperature until subsequent genetic analysis at the Southwest Fisheries Science Center (SWFSC), La Jolla, California, and the University of Florida's Whitney Laboratory for Marine Bioscience, St. Augustine, Florida, and the skull was removed and macerated in cold water in order to examine diagnostic features of the skeletal anatomy. Multiple samples of tissue were collected from a single large tumor (Fig. 3) present on the ventral side of the right front flipper using 6-mm biopsy punches during necropsy. Tumor tissue samples were preserved frozen at -80°C until analysis. Histopathology was not performed due to decomposition and prior freezing.

DNA Extraction.—Genomic DNA was extracted from a skin sample following a NucleoMag® Tissue Kit extraction protocol (Macherey-Nagel, #744300.4) at SWFSC. An ~800-bp fragment of the mitochondrial DNA control region was amplified using polymerase chain reaction (PCR) methodologies with standard PCR reagents and primers LCM-15382 (5' GCT TAA CCC TAA AGC ATT GG 3') and H950g (5' GTC TCG GAT TTA GGG GTT TG 3'; Abreu-Grobois et al. 2006). Both strands were cycle sequenced with an ABI® Big Dye™ Terminator v3.1 Cycle Sequencing Kit and analyzed with Applied Bio-systems® (model 3730) automated genetic analyzer.

DNA was extracted from a non-tumored (skin) tissue sample and a tumored tissue sample using a DNeasy Blood & Tissue Kit (Qiagen, #69504). The whole genome sequencing libraries were prepared and sequenced at the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida, Gainesville, Florida, as previously described (Yetsko et al. 2020). The sequencing libraries were constructed using the NEBNext UltraTMII DNA Library Prep Kit for Illumina (#E7645S). Paired-end sequencing was conducted on an Illlumina NovaSeq 6000 with a read length of 150 bp.

ChHV5 Sequencing.—The sequenced library for the tumored tissue sample (sample ID: LoTXFdna) was uploaded to Galaxy for analysis (https://usegalaxy.eu/), and its quality inspected, using the FastQC tool. The DNA-Seq data including raw reads are deposited in NCBI (https://www.ncbi.nlm.nih.gov/) under BioProject ID: PRJNA449022. Adapters were trimmed and any low-quality reads removed using Trimgalore. Sequences were then aligned to the ChHV5 genome (Ackermann et al. 2012; NCBI accession number: HQ878327.2), using Bowtie2. The Ococo consensus caller (https://arxiv.org/abs/1712.01146) was used to generate a consensus sequence from the ChHV5 reads (Bowtie2 alignment output BAM file) compared to the ChHV5 reference sequence. ChHV5 viral sequences specific to the UL30 gene were extracted using the SMS Range Extractor DNA tool (https://www.

Table 1. GenBank accession numbers, sequence IDs, and sampling locations of *Caretta caretta* (Cc), *Chelonia mydas* (Cm), and *Lepidochelys olivacea* (Lo) chelonid alphaherpesvirus 5 (ChHV5) UL30 samples used in the present study.

Accession #	Name in Tree (Fig. 5)	Species	Location
HM348897.1	ChHV5 UL30 AtGG 462	Cm	Príncipe Island
JN938585.1	ChHV5 UL30 AtBR 483	Cm	Brazil
JN580279.1	ChHV5 UL30 AtPR2 473	Cm	Puerto Rico
JN580280.1	ChHV5 UL30 AtPR 473	Cm	Brazil
JN938586.1	ChHV5 UL30 AtBR 483 var3	Cm	Brazil
JN938587.1	ChHV5 UL30 AtBR 483 var4	Cm	Brazil
JN580283.1	ChHV5 UL30 AtPR6 473	Cm	Puerto Rico
AF299107.1	ChHV5 UL30 PaAUS 483	Cc	Brisbane, Australia
HQ878327.2	Original genome UL30 483	Cm	Hawaii
AY646893.1	HA variant UL30	Cm	Hawaii
AF299110.1	ChHV5 UL30 AtCAR 483	Cm	Barbados
AY646888.1	ChHV5 UL30 varA AtFL 483	Cm	Florida
AY646889.1	ChHV5 UL30 varC AtFL 483	Cm	Florida
AY646892.1	ChHV5 UL30 varB AtFL 483	Cm	Florida
LoTXFdna	LoTXFdna UL30	Lo	Texas
AY646890.1	FL variant D UL30	Cm	Florida
AY390422.1	ChHV5 UL30 PaCA 401	Cm	California
AF299109.1	ChHV5 UL30 AtMEX 483	Cm	California

bioinformatics.org/sms2/range_extract_dna.html). The sequence of UL30 was compared to previously published ChHV5 sequences for that gene (https://www.ncbi.nlm.nih.gov/; Table 1) for phylogenetic analysis, using MEGA-X (https://www.megasoftware.net/). Phylogenetic trees were constructed using the Maximum Likelihood method in the Tamura-Nei model, with all other input parameters kept to default.

Results

The stranded sea turtle measured 68.4 cm SCL_{max}, 67.5 cm SCL_{min}, and 57.0 cm SCW. The plastron had four pairs of inframarginal scutes, but a count of the central and costal scutes was not possible due to decomposition. The turtle was suspected to be an Olive Ridley Sea Turtle based on general shape of the carapace and skull. The presence of pronounced pterygoid processes was indicative of Olive Ridley and not Kemp's Ridley Sea Turtles (Wyneken 2003). Species was confirmed to be Olive Ridley Sea Turtle by genetic analysis of skin tissue. The sequence produced at the SWFSC was edited and aligned using the program Geneious R8.1.9 (Kearse et al. 2012), which compares the sequence to a local database of published and unpublished haplotypes. The sequence was also compared to sequences published on GenBank using a nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the species and haplotype. The results matched haplotypes found in western Atlantic (French Guiana) Olive Ridley Sea Turtle populations (Genbank Accession ID FJ795404–433 in Plot et al. 2012).

The neck and front flippers were entangled in monofilament line, which was also wrapped around the base of a large tumor emanating from the ventral aspect of the right front flipper. The tumor measured 8 x 10 x 5 cm and was pedunculated with a broad base. The surface was diffusely ulcerated and on section it was solid, firm, and white, consistent with a fibropapilloma. During necropsy, the turtle was determined to be in fair nutritional condition and cause of death was attributed to drowning by entanglement. The internal organs were severely decomposed; however, *Pennatulacea* were found in the esophagus and the discernable portions of the colon. *Pennatulacea* are a favored food item of Loggerhead Sea Turtles inhabiting south Texas waters, but not Kemp's Ridley Sea Turtles documented there (Shaver 1991; Plotkin et al. 1993). The sex was confirmed to be female based on the presence of immature oviducts. The presence of ChHV5 in the Olive Ridley Sea Turtle's tumor tissue was confirmed by next-generation sequencing. The non-tumored skin sample also tested positive for ChHV5 DNA; however, there were over 100 times more ChHV5 DNA reads present in the FP tumor sample than in the non-tumored skin sample (Fig. 4). A 483-bp and a 397-bp sequence fragment of the UL30 gene, which encodes a DNA polymerase, were used for phylogenetic comparison with published ChHV5 variants (Fig. 5A–C; Table 1). The ChHV5 variant in the Olive Ridley Sea Turtle's FP tumor most closely grouped to the ChHV5 A–C variants from Florida's Atlantic coast (Fig. 5A–C; Table 1).

Discussion

We document the first reported stranding of an Olive Ridley Sea Turtle on the western GoM coast, USA, and confirm the sixth sea turtle species to strand on the Texas coast (Shaver 1994). The Olive Ridley Sea Turtle was found entangled in monofilament line. Discarded fishing equipment was associated with 12.4% of dead stranded sea turtles (excluding dead stranded turtles identified as cold stunned) documented on the Texas coast from 1980–2019 (D. Shaver, unpubl. data). Entanglement in fishing nets was the cause of stranding for an Olive Ridley Sea Turtle documented near south Florida (Foley et al. 2003) and an Olive Ridley Sea Turtle sighted near Puerto Rico (Horta et al. 2000).

The first case of FP in Texas was confirmed in 2010 (Tristan et al. 2010; Shaver et al. 2019) and has significantly increased in Green Sea Turtles in south Texas since 2015 (Shaver et al. 2019). Much of the published information available on FP and Olive Ridley Sea Turtles is from the Pacific population, although the disease has been documented in Olive Ridley Sea Turtles found stranded in Brazil (D. W. Goldberg, pers comm.). Although we were not able perform histopathology due to decomposition, the gross features of the tumor found on the Texas-stranded Olive Ridley Sea Turtle were typical of FP. In addition, the ChHV5 load within the FP tumor was >100 times higher than in its non-tumored skin sample, similar to the results of whole genome sequencing-based viral-load quantification in Green Sea Turtles (Yetsko et al. 2020). Phylogenetic analysis revealed that the Texas-stranded Olive Ridley Sea Turtle's ChHV5 UL30 gene grouped most closely with that of Atlantic/GoM samples, particularly Florida variants A to C (Fig. 5A). Florida variants A, B, and C are known to be almost identical, with the D variant the most dissimilar of the four (Herbst et al. 2004). To further investigate their relatedness, a phylogenetic tree consisting of just the Florida variants A to C and the Texas Olive Ridley Sea Turtle sample was produced (Fig. 5B). The UL30 sequence from the Texas Olive Ridley Sea Turtle was most closely related to Florida variant B, although all four sequences are extremely closely clustered.

Chilean and Baja Californian Olive Ridley Sea Turtle ChHV5

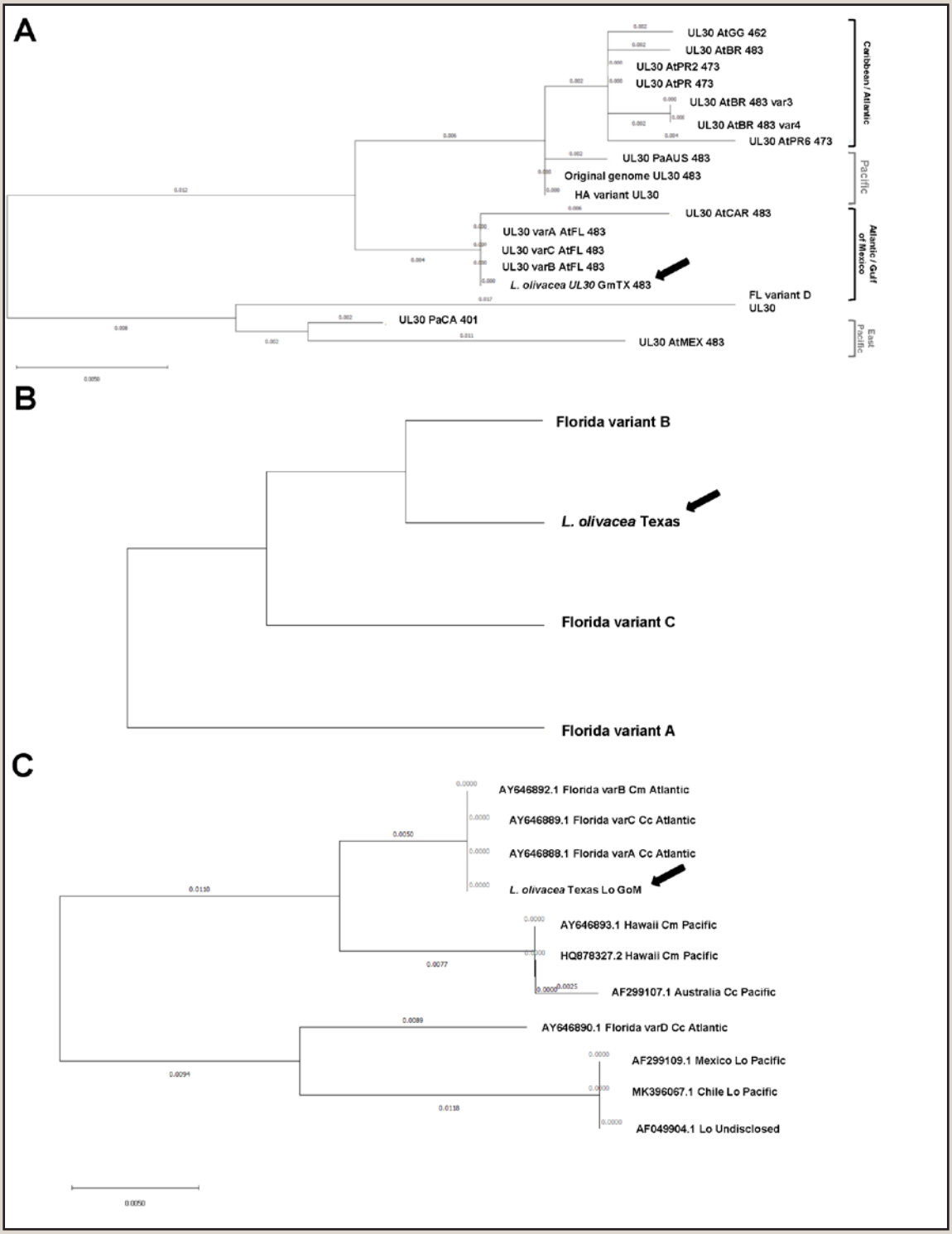

FIG. 5. Phylogenetic relationship between the chelonid alphaherpesvirus 5 (ChHV5) viral variant isolated from the Olive Ridley Sea Turtle (*Lepidochelys olivacea*) and other known variants: A) Phylogenetic tree generated from 18 aligned ChHV5 UL30 gene sequences (483 bp). Individual samples are identified by their ChHV5 viral gene and strain and oceanic region (At = Atlantic; Pa = Pacific) followed by geographic coast in which turtle samples were collected (FL = Florida; TX = Texas; AUS = Australia; CAR = Caribbean; GG = Gulf of Guinea; BR = Brazil; PR = Puerto Rico; HA = Hawaii; MEX = Mexico; CA = California); B) Phylogenetic tree generated from 4 aligned ChHV5 UL30 gene sequences (483 bp). Individual samples are identified by variant within the state where they were collected; C) Phylogenetic tree generated from 11 aligned ChHV5 UL30 gene sequences (397 bp), from three species– *Caretta caretta* (Cc), *Chelonia mydas* (Cm) and *Lepidochelys olivacea* (Lo). The ChHV5 sequence from the *L. olivacea* stranded in Texas, from the present study, is highlighted with a black arrow. Branch figures represent numbers of substitutions per site, along with scale bar.

sequences (UL27, UL28 and UL30) clustered with the Eastern Pacific group (Álvarez-Varas et al. 2019; Espinoza et al. 2020) and a Costa Rica Olive Ridley Sea Turtle's ChHV5 sequences (glycoprotein B gene) clustered with ChHV5 from a Green Sea Turtle that stranded in San Diego, California, USA (Greenblatt et al. 2005; Jones et al. 2020). Olive Ridley Sea Turtle ChHV5 sequences from an unspecified population formed a separate clade to early Hawaii and Florida ChHV5 sequences (Quackenbush et al. 1998). When the UL30 ChHV5 sequence from the Olive Ridley Sea Turtle stranded in Texas was compared with these other Olive Ridley Sea Turtle ChHV5 UL30 sequences, it did not group with those samples but continued to cluster with the Florida variants A to C from Green and Loggerhead Sea Turtles (Fig. 5C). Our data further confirm that regional ChHV5 variant grouping occurs, that variants are independent of the marine turtle species (i.e., they have not arisen by host species-virus coevolution), and that cross-species transmission of ChHV5 occurs.

Individual sightings of Olive Ridley Sea Turtles in USA waters of the Atlantic and GoM should not be taken as an indication of range expansion for a species; rather, these observations serve as a reminder of the relatively broad connectivity of sea turtle populations. The Olive Ridley Sea Turtle found in Texas was afflicted by FP and succumbed to entanglement in fishing line, both of which are known threats to marine turtles in the southeastern USA. Distant movements of individuals beyond their core range, even as relatively rare events, can potentially disseminate pathogens, such as variants of ChHV5.

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Seasonal Shift in the Age Structure of Calling Males Within a Spring Peeper (*Pseudacris crucifer*) Chorus

Many factors can influence male reproductive success including attractiveness of sexual displays, effectiveness of sensory and locomotory systems in scramble competition, physical condition in fight contests, or quality of resources offered to mates. In lek breeding as is found for many species of anurans, the ability to remain reproductively active within a breeding assemblage is one of the most important predictors of mating success (Halliday and Tejedo 1995; Andersson and Iwasa 1996; Friedl and Klump 2005; Castellano et al. 2009). Males of many anuran species congregate in wetlands and produce advertisement calls to attract females for reproduction. Males that spend more nights calling in a breeding chorus than average (hereafter chorus tenure) tend to have greater mating success (Greer and Wells 1980; Sherman 1980; Halliday and Tejedo 1995; Friedl and Klump 2005; Mangold et al. 2015; Botto and Castellano 2016). A positive relationship between chorus tenure and mating success is evident for most taxa where this has been evaluated (Table 1). Chorus tenure often surpasses all other factors in explaining male mating success variance, such as attributes of advertisement calls and body size that are often assumed to play a key role in sexual selection (Sullivan and Hinshaw 1992; Friedl and Klump 2005; Castellano et al. 2009; Ospina-L. et al. 2017). For example, chorus tenure explained about 50% of the variance in mating success in Natterjack Toads, *Epidalea calamita* (Arak 1983) and European Treefrogs, *Hyla arborea* (Jaquiéry et al. 2010). In Italian Treefrogs, *Hyla intermedia*, chorus tenure explained 19% of mating success variance, while call rate, one of the most important call attributes under direct female selection in the field, accounted for only 5% (Castellano et al. 2009).

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Abbreviated chorus tenure, when males spend only a fraction of breeding season in a chorus, has been observed in many anuran species (Murphy 1994b): for example, over 50% of males called only 1-2 nights and the maximum chorus tenure was only 13 nights over a single 4-mo breeding season in Rosenberg's Treefrog, *Hypsiboas rosenbergi*, in Puntarenas Province, Costa Rica (Höbel 2000). The median chorus tenure was also only 2–3 nights in the Barking Treefrog, *Dryophytes gratiosus*, in southwest Tallahassee (Florida, USA), whose breeding season lasted 49–96 nights from 1987 to 1990 (Murphy 1994a). Even for Great Plains Toads, *Anaxyrus cognatus*, whose breeding season was only 2–5 nights, over 90% males called for only a single night in southern Arizona and southwestern New Mexico, USA from 1980 to 1982 (Sullivan 1983). Non mutually-exclusive hypotheses for variance in male chorus tenure that have at least some empirical support include: 1) the energy limitation hypothesis proposing that some males cannot sustain costly calling behaviors for many nights (Green 1990; Murphy 1994b); 2) the predation risk hypothesis where males choose to leave choruses due to high predation risk (Green 1990); and 3) the mortality hypothesis where males are removed from choruses because of predation, parasitism, disease or desiccation (Murphy 1994b). To test the energy limitation hypothesis, feeding starvation experiments are commonly done to test whether feeding increases the number of nights males call, and male body size and body condition are examined to test whether they are correlated with chorus tenure (Green 1990; Murphy 1994b; Given 2002). In some species, smaller males lost weight more quickly than larger males and thus attended their respective chorus for shorter periods of time (e.g., Natterjack Toads; Tejedo 1992). In contrast, Morrison et al. (2001) found that smaller male Orange-thighed Frogs, *Litoria xanthomera*, of northern Queensland, Australia, attended choruses for longer due to slower energy consumption. In some species only body size predicts chorus tenure, but not body condition (e.g., European Treefrogs; Luquet et al. 2013), while in other species body size does not relate to chorus tenure at all (Arak 1988; Woodward 1982; Dyson et al. 1992; Given 2002; Grafe and Meuche 2005; Basto-Riascos et al. 2017). Thus, the effect of body size and body condition on chorus tenure appears to be species-specific. Empirical data for the predation risk hypothesis and the mortality hypothesis