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COMMUNICATIONS

Detection of Green Turtle Herpesviral Sequence in Saddleback Wrasse *Thalassoma duperrey*: A Possible Mode of Transmission of Green Turtle Fibropapilloma

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Abstract.—Samples of DNA were prepared from various tissues and organs (including snout, gill, eye, brain, heart, liver, gut content, intestine, swim bladder, spleen, gallbladder, spinal cord, gonad, and muscle) of six healthy appearing reef cleaner fish, saddleback wrasses *Thalassoma duperrey*, captured from a cleaning station in North Kaneohe Bay, Oahu, Hawaii. The DNA samples were tested for evidence of green turtle herpesvirus infection by nested polymerase chain reaction (PCR). Green turtle herpesviral sequences were detected in snout (3/6), gill (2/6), and liver (1/6). All other tissues were negative. Except for a single nucleotide substitution (from A to G at position 48, resulting in a single amino acid change from isoleucine to methionine at position 16), the DNA sequences detected in the fish were identical to that of a newly reported green turtle herpesvirus. Although preliminary, these data represent the first evidence for an association of a herpesvirus with saddleback wrasse, suggesting that cleaner fish may serve as vectors or carriers for the transmission of the agent causing green turtle fibropapilloma.

The near-epidemic occurrence of fibropapilloma has threatened the survival of the green turtle *Chelonia mydas*, a marine reptile protected under the provision of the U.S. Endangered Species Act (Balazs and Pooley 1991; Herbst 1994; George 1997). Although the etiologic agent has yet to be identified, mounting evidence suggests that green turtle fibropapilloma (GTFP) results from a viral infection (Herbst et al. 1995; Aguirre and Spraker 1996; Casey et al. 1997; Quackenbush et al. 1998; Lu et al. 1999), and a herpesvirus has been implicated (Aguirre and Spraker 1996; Quackenbush et al. 1998). Whereas attempts at isolating and characterizing the etiologic virus of GTFP are urgently needed, equally important are studies aimed at clarifying the transmission of GTFP among green turtles in their natural habitats. In this regard, cleaning symbiosis between green turtles and various reef fish has been widely documented (Booth and Peters 1972; Hubbs 1977; Frazier et al. 1985; Smith 1988; Losey et al. 1994; Balazs 1996). In particular, some reef fish are known to be highly specialized cleaner species that feed on organisms growing on the surfaces of green turtles.

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Because green turtles are frequent visitors to reef cleaning sites and because reef cleaners are commonly observed biting tumors of turtles (Losey et al. 1994; Zamzow 1999), we hypothesized that reef cleaner fish may serve as carriers or vectors for the transmission of GTFP (Losey et al. 1994). That is, by causing skin wounds that leave turtles more vulnerable to infection and by moving from one turtle to the next, cleaner fish may horizontally transfer the presumptive virus of GTFP. No study has been conducted to examine reef cleaners for viruses, and little is known about their role in the transmission and spread of GTFP. In this study, we report the detection of green turtle herpesviral sequences in the saddleback wrasse *Thalassoma duperrey*.

Methods

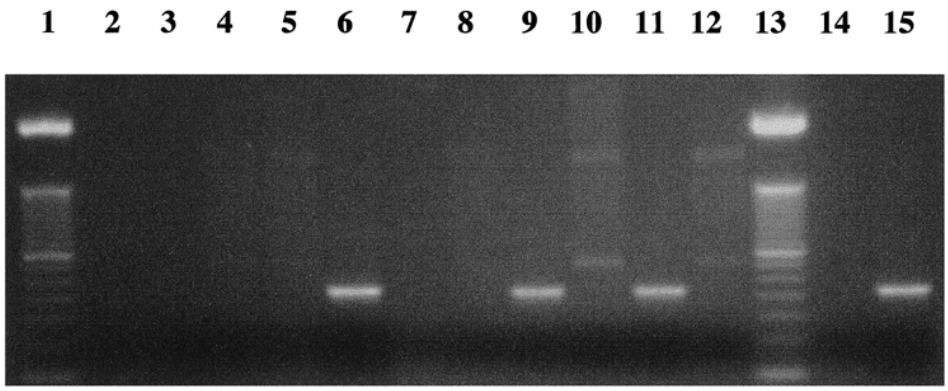
Six saddleback wrasses were captured by spearing or with baited hook and line between August and September 1998 during a monitoring period at a green turtle cleaning station, at reef 42 in North Kaneohe Bay (21.5°N, 158.8°W) of the island of Oahu, Hawaii. As controls, 11 saddleback wrasses were collected from Checker Reef, a noncleaning site at South Kaneohe Bay, where no turtles were observed either on the surface or in the water. Fish, measuring 13–17 cm in body length, were kept on wet ice for 3–4 h during their transport to the laboratory. These reef wrasses were euthanatized and their gut contents were examined for turtle-specific skin barnacles *Platylepas hexastylos* at 60× magnification according to a previously described method (Losey et al. 1994). Tissues and organs—including snout, gill, eye, brain, heart, liver, gut content, intestine, swim bladder, spleen, gallbladder, spinal cord, gonad, and muscle—were stored in a −70°C freezer after dissection using separate sterile surgical scissors, forceps, and knives.

For DNA extraction, 0.3–0.5 g of each tissue was ground into a fine powder using a prechilled mortar and pestle and suspended in digestion buffer (100 mM NaCl; 10 mM tris–HCl, pH 8.0; 25 mM EDTA, pH 8.0; 0.5% sodium dodecyl sulfate and 0.1 mg proteinase K/mL) at an approximate ratio of 100 mg tissue/1.2 mL digestion buffer. Suspensions were then transferred into tightly capped 50-mL polypropylene tubes and incubated overnight (15–18 h) at 50°C. Digests were deproteinized by successive phenol–chloroform–isoamyl alcohol extractions, and DNA was recovered by ethanol precipitation, then dried and resuspended in 100–300 μL TE buffer (10 mM tris–HCl, 1 mM EDTA, pH 8.0). Nucleic acid concentrations were determined by spectrophotometry at 260 Å, and DNA quality was confirmed by electrophoresis on 1% agarose gels.

Detection of green turtle herpesviral sequences in fish tissues was performed by nested polymerase chain reaction (PCR) using two oligonucleotide primer pairs designed from the highly conserved region of the herpesviral DNA-directed DNA polymerase gene (GenBank Accession number AF035003) reported from green turtles (Quackenbush et al. 1998). The forward and reverse primers for the primary PCR were 5′-AGCATCATCCAGGCCCACAATCT-3′ and 5′-CGGCCAGTTCCGGCGCGTCGACCA-3′, respectively (A = adenine, G = guanine, C = cytosine, T = thymine), which produced a PCR product of approximately 445 base pairs (bp). Fish DNA (2 μL) was amplified in 25-μL reaction mixtures containing 0.1 μM each PCR primer, 200 μM each dNTP (deoxynucleotide triphosphate), 2 mM $MgCl_2$ and 1.25 U *Taq* polymerase. Following an initial denaturation for 5 min at 94°C, reaction mixtures were cycled 5 times at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and then cycled 45 times at 94°C for 30 s, 50°C for 30 s and 72°C for 60 s in a Perkin-Elmer GeneAmp PCR System 2400 using 0.2-mL thin-wall snap-cap tubes. After cycling, reaction mixtures were incubated for 7 min at 72°C for the final extension, then held at 4°C. Nested PCR was performed on 2 μL of the primary PCR amplicon in a 25-μL reaction volume with upstream primer 5′-CTGCTGACCGACTGGC-TGGC-3′ and downstream primer 5′-AGCATG-TCGCGCCCTACGGTGGTGAC-3′ employing the following reaction conditions: 1.5 min denaturation at 94°C, followed by 10 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, then 45 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. Amplification was performed with the inclusion of DNA prepared from a heart tumor of a green turtle and nucleic-acid-free water as positive and negative PCR controls, respectively. The PCR products were size fractionated on 2% NuSieve agarose gels and a DNA fragment of approximately 206 bp was considered positive.

Representative amplicons of the expected size were cloned into the TA vector (pCR2.1; original TA cloning kit, Invitrogen), and clones were sequenced in both directions using the same oligonucleotides employed in nested PCR. Four randomly selected clones were sequenced for each posi-

A



B

CTGCTGACCG ACTGGCTGGC ACTCAGGAAA AAAATCCGCG CATCGATGAA
AACCGCACCG AGCGACCAAC GCTTGCTTTT GGACAAGCAG CAGCTGGCCA
TCAAGCTGAC GTGCAATTCC GTTTACGGGT TTACCGGCGT GGCCACCGGA
TTCCTACCTT GTCTGGAGGT GGCGGCCACG GTCACCACCG TAGGGCGCGA
CATGCT

FIGURE 1.—**(A)** Amplification, by nested polymerase chain reaction (PCR), of herpesviral sequence in DNA extracted from tissues and organs of saddleback wrasse; fragment size was estimated using molecular size markers, and a 206-bp fragment indicated the presence of herpesvirus. Lanes 1 and 13 are 50-bp DNA ladder; interior lanes are fish intestine (2), swim bladder (3), nerve (4), gut content (5), snout (6), eye (7), kidney (8), liver (9), brain (10), gill (11), and muscle (12); negative control is water (14), and positive control is green turtle eye tumor (15). **(B)** Nucleotide sequence of the 206-bp herpesviral fragment. Underlined portions represent positions of the nested PCR primers.

tive amplicon. Sequence analysis was facilitated using several computer programs, including DNASTAR.

For comparative analysis, a radial phylogenetic neighbor-joining tree of DNA polymerase gene sequences of different herpesviruses was generated with the SeqPup/PPC program. Percent frequencies of the groupings were determined after 1,000 bootstrap evaluations.

Results and Discussion

To determine the potential role of reef cleaner fish in the occurrence of GTFP, tissues of reef cleaner and noncleaner saddleback wrasses from Kaneohe Bay were examined for green turtle herpesviral sequences by nested PCR. Cleaning activity of reef fish was ascertained by underwater remote-video cinematography and by the presence of turtle-specific skin barnacles *P. hexastylos* in their gut contents. Under the described PCR conditions, amplification resulted in a distinct fragment measuring 206 bp (Figure 1). Of the six cleaner saddleback wrasses, green turtle herpesviral sequences were detected from the snouts of three (50%), the gills of two (33%) and the liver of one (17%) fish (Table 1). All other tissues from

TABLE 1.—Summary of PCR[a] detection of green turtle herpesviral sequence in tissues of 6 cleaner and 11 noncleaner reef fish (saddleback wrasses). Values indicate number of fish in which the tissue sample was positive for DNA of the virus; ND = PCR not done.

Tissue	Cleaners	Noncleaners
Snout	3	0
Eye ball	0	ND
Gill	2	0
Brain	0	ND
Heart	0	ND
Spleen	0	ND
Liver	1	0
Gall bladder	0	ND
Gonad	0	ND
Swim bladder	0	ND
Spinal cord	0	ND
Intestine	0	ND
Kidney	0	ND
Nerve	0	ND
Muscle	0	ND
Gut content	0	ND

[a] Polymerase chain reaction.

these six cleaning wrasses and the snout, gill, and liver tissues from the 11 noncleaner wrasses tested negative for the herpesviral sequence.

Examination of the distribution of the six positive tissues for herpesvirus showed that the viral sequence was only detected in three of six fishes. Among these three saddleback wrasses, green turtle herpesvirus in cleaner fish (GTHV-cf) was present in snout, gill, and liver of fish number 2, in snout and gill of fish number 3, and in snout of fish number 4. Variation in detection of green turtle herpesviral sequence among these cleaner fish may reflect diverse interactions between each individual fish and turtles during the cleaning event. Detection of the herpesviral sequence in external snout and gill could result from physical contact of cleaner fish with turtle tumors during cleaning, whereas finding of the herpesvirus in liver tissue may suggest the occurrence of viral infection. We are currently investigating the natural association of the herpesvirus with cleaner fish by examining additional fish from various cleaning sites in the Hawaiian Islands and by using in vitro cell culture to isolate the virus.

Genetic analysis of representative clones indicated sequence identity between different tissues of the fish (data not shown). Comparative analysis of the herpesviral sequences in the fish tissues indicated near identity with that of green turtle herpesvirus (GTHV) detected in green turtles with fibropapilloma (Figure 2). Moreover, except for a single nucleotide substitution from A (turtle) to G (fish) at position 48, which resulted in a single amino acid change from isoleucine (turtle) to methionine (fish), the fish and turtle sequences were identical. The functional significance, if any, of this point mutation is unknown.

Cleaning symbiosis between various aquatic organisms has been frequently referenced in the literature (Limbaugh 1961; Hubbs 1977; Balazs 1980, 1996; Gorlick 1980; Boschung et al. 1983; Frazier et al. 1985; Losey 1987; Smith 1988; Losey et al. 1994; Moll et al. 1995; Balazs 1996). Most reports document interspecific interactions between marine fish, and a few descriptions involve fish and crustaceans (Smith 1988). Reported in 1972, early observations of the cleaning of marine turtles in the wild involved two species of reef fish, moon wrasse *Thalassoma lunare* and damselfish *Abudefduf sexfasciatus*, feeding on the skin surface of green turtles (Booth and Peters 1972). The cleaning behavior of reef fish has also been documented on the hawksbill turtle *Eretmochelys imbricata* (Smith 1988). Some cleaners were observed to have an apparently specialized cleaning relationship with green turtles in Hawaii (Losey et al. 1994; Balazs 1996). In 1994, Losey and colleagues observed some individual saddleback wrasses specializing both their diet and behavior to exploit not only parasitic barnacles but also tumors on green turtles in Hawaii (Losey et al. 1994). A recent statistical analysis indicated that the percentage of turtle tumors bitten by saddleback wrasses was more than 90% at several cleaning sites around the Hawaiian Islands (Zamzow 1999).

Close and frequent contact between cleaner and host species creates ample opportunity for transfer of infectious organisms. Hobson reported that the California cleaning wrasses, including senorita *Oxyjulis californica*, sharpnose seaperch *Phanerodon atripes* and kelp perch *Brachyistius frenatus*, were frequently infected by the same ectoparasites as the host species on which it most often feeds (Hobson 1971). Our findings support the notion that the saddleback wrasse may function as a vector in the spread of GTFP among green turtles. By video-camera recording (Losey et al. 1994; Zamzow 1999), these cleaner fish were observed to move between green turtles and to bite tumors of affected green turtles. The PCR data indicated that herpesviral sequences were present in as much as 50% of snout and 33% of gill tissues from cleaner fish ($N = 6$). Our preliminary data suggest that the cleaner fish might serve to mechanically transmit the presumptive etiologic virus of GTFP. However, further studies are warranted to elucidate the

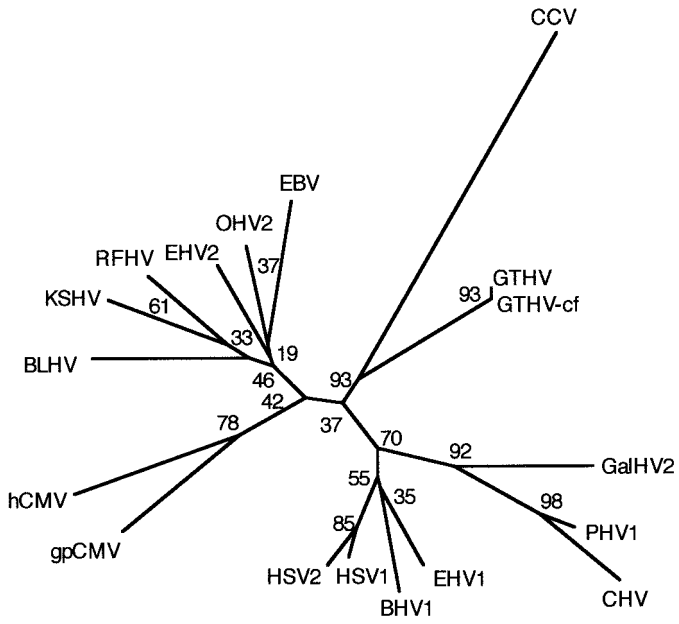


FIGURE 2.—A radial phylogenetic neighbor-joining tree of DNA polymerase gene sequences (206 bp) of different herpesviruses. Numbers at each branch indicate frequency (%) of this grouping after 1,000 bootstrap evaluations. Channel catfish virus was used as an outgroup. Viruses are abbreviated as follows.

BHV-1, bovine herpesvirus 1
BLHV, bovine lymphotropic herpesvirus
CCV, channel catfish virus
CHV, canine herpesvirus
EBV, Epstein–Barr virus
EHV-1, equine herpesvirus 1
EHV-2, equine herpesvirus 2
GalHV-2, gallid herpesvirus 2
gpCMV, guinea pig cytomegalovirus
GTHV, green turtle herpesvirus
GTHV-cf, green turtle herpesvirus in cleaner fish
hCMV, human cytomegalovirus
HSV-1, herpes simplex virus 1
HSV-2, herpes simplex virus 2
KSHV, Kaposi's sarcoma-associated herpesvirus
OHV-2, ovine herpesvirus 2
PHV-1, phocine herpesvirus 1
RFHV, retroperitoneal fibromatosis-associated herpesvirus

pathogenesis of herpesviral infection in green turtles and to determine whether reef cleaners are actually infected with this virus.

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