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Discovery of a Novel Single-Stranded DNA Virus from a Sea Turtle Fibropapilloma by Using Viral Metagenomics▽

Terry Fei Fan Ng,[1] Charles Manire,[2]† Kelly Borrowman,[3] Tammy Langer,[4]‡ Llewellyn Ehrhart,[3] and Mya Breitbart[1]*

College of Marine Science, University of South Florida, 140 7th Avenue South, St. Petersburg, Florida 33701[1]; Mote Marine Laboratory, 1600 Ken Thompson Parkway, Sarasota, Florida 34236[2]; College of Sciences, University of Central Florida, 4000 Central Florida Blvd., Orlando, Florida 32816-2368[3]; and Clearwater Marine Aquarium, 249 Windward Passage, Clearwater, Florida 33767[4]

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Viral metagenomics, consisting of viral particle purification and shotgun sequencing, is a powerful technique for discovering viruses associated with diseases with no definitive etiology, viruses that share limited homology with known viruses, or viruses that are not culturable. Here we used viral metagenomics to examine viruses associated with sea turtle fibropapillomatosis (FP), a debilitating neoplastic disease affecting sea turtles worldwide. By means of purifying and shotgun sequencing the viral community directly from the fibropapilloma of a Florida green sea turtle, a novel single-stranded DNA virus, sea turtle tornovirus 1 (STTV1), was discovered. The single-stranded, circular genome of STTV1 was approximately 1,800 nucleotides in length. STTV1 has only weak amino acid level identities (25%) to chicken anemia virus in short regions of its genome; hence, STTV1 may represent the first member of a novel virus family. A total of 35 healthy turtles and 27 turtles with FP were tested for STTV1 using PCR, and only 2 turtles severely afflicted with FP were positive. The affected turtles were systemically infected with STTV1, since STTV1 was found in blood and all major organs. STTV1 exists as a quasispecies, with several genome variants identified in the fibropapilloma of each positive turtle, suggesting rapid evolution of this virus. The STTV1 variants were identical over the majority of their genomes but contained a hypervariable region with extensive divergence. This study demonstrates the potential of viral metagenomics for discovering novel viruses directly from animal tissue, which can enhance our understanding of viral evolution and diversity.

Discovering novel viruses is difficult due to the inability to grow many viruses in cell culture systems, the small size and low nucleic acid content of viruses, and the lack of a conserved genetic element found in all viral genomes. The most common methods for diagnosing viral infections use PCR, microarrays, immunologic assays, or replication in cell culture to test for specific viruses. These methods are limited for viral discovery because many viruses cannot be cultured on standard cell lines, and primers and panviral microarrays require some degree of sequence conservation in order to identify viruses. Recent studies have demonstrated that viral particle purification and shotgun sequencing (viral metagenomics) constitute an effective method for describing novel viruses, including those that are unrelated to previously described viral families (reviewed in references 23 and 28). Viral metagenomics has been used to characterize viral communities present in the environment (6, 8, 11, 15, 22, 24, 25, 31) and to describe viruses in mammal feces (12, 13, 16, 69), cell culture (39, 43), respiratory tract aspirates (3), and blood (14, 40). However, to date, viruses have not been purified directly from animal tissues for metagenomic sequencing, due to the high background concentrations of host nucleic acids. In this study, we successfully purified viruses directly from a sea turtle fibropapilloma and subsequently discovered a novel virus that could not have been identified using PCR with degenerate primers or a panviral microarray. The methods presented here are directly applicable to a variety of animals and tissue types, therefore significantly enhancing our ability to discover novel viruses.

Fibropapillomatosis (FP) is a debilitating neoplastic disease affecting sea turtles worldwide (2, 34). Affected turtles develop epithelial fibropapillomas, and some turtles develop internal fibromas in terminal cases (67). FP primarily affects green sea turtles, *Chelonia mydas*, but has been confirmed in all seven sea turtle species (32, 35). In some regions, such as the Indian River Lagoon in Florida, FP affects more than half of the green turtle population (19, 32, 34). FP has been documented since the late 1930s (61) and is now widespread throughout the world (32, 35). Since all species of marine turtles are either endangered or threatened (18), the additional stress of FP poses a potential threat to the future survival of sea turtles.

The etiological agent responsible for FP is unknown; however, current evidence strongly supports the involvement of an alphaherpesvirus, FP-associated turtle herpesvirus (FPTHV) (45, 47, 54, 55, 68). Transmission studies have demonstrated that the infectious FP agent is less than 0.45 μm in size and can be inactivated by organic solvents and high-speed centrifugation, which is consistent with the theory of an enveloped virus as the causative agent (36, 37). Using PCR, FPTHV DNA has been found in most examined fibropapillomas but not detected in the vast majority of healthy turtles (45, 47, 54). However, extensive attempts at isolating and propagating FPTHV in cell

* Corresponding author. Mailing address: University of South Florida, College of Marine Science, 140 7th Avenue South, St. Petersburg, FL 33701. Phone: (727) 553-3520. Fax: (727) 553-1189. E-mail: mya@marine.usf.edu.
† Present address: Atlantis Resort, Paradise Island, Bahamas.
‡ Present address: Volusia Marine Science Center, Ponce Inlet, FL 32127.
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TABLE 1. PCR primers used to sequence the STTV1 genome, characterize STTV1 prevalence, and determine genome polarity and strandedness

Primer name	Sequence (5′ to 3′)	PCR conditions
STTV1-a Forward	GAGTATTGCCGAGGCTCAAG	95°C for 5 min; 45 cycles of [94°C for 30 s, 58°C minus 0.2°C per cycle for 30 s, 72°C for 2 min]; 72°C for 10 min
STTV1-a Reverse	TGCAGAATTCTCGTTGAAGG	
STTV1-b Forward	AGTCAGAGAGCAGCGGATGT	Same as for STTV1-a
STTV1-b Reverse	ATGGTACAACCCCAAAGCG	
STTV1-c Forward	AAGCACCTTCCCAATACGG	95°C for 5 min; 45 cycles of [94°C for 30 s, a gradient of annealing temp of 55.4°C, 58.1°C and 60.7°C minus 0.2°C per cycle for 30 s, and 72°C for 2 min]; 72°C for 10 min
STTV1-c Reverse	TTATAGGGGCAGTAGGCACG	
STTV1-d Antisense	ACATCCGCTGCTCTCTGACT	95°C for 5 min; 50 cycles of [94°C for 1 min, 49°C for 1 min, 72°C for 3 min]; 72°C for 10 min
STTV1-d Sense	GGGAGGATTGCTGAGATGAA	
STTV1-e Forward	CGTGCCTACTGCCCCTATAA	Same as STTV1-d
STTV1-e Reverse	CCAGATAATTAGTAGCACCAGAATGA	
STTV1-e Forward	CGTGCCTACTGCCCCTATAA	95°C for 5 min; 45 cycles of [94°C for 1 min, 54°C minus 0.2°C per cycle for 1 min, 72°C for 3 min]; 72°C for 10 min
STTV1-f Reverse	GGGAGGATTGCTGAGATGAA	

culture have been unsuccessful (19). Since FPTHV has not been obtained in pure culture and shown to cause FP, it is possible that there are other undescribed, coexisting viruses involved in the etiology of FP. For example, Lu et al. (48) observed a small naked virus able to induce tumor-like aggregates in sea turtle cell lines. Only a very short sequence was obtained from these viruses, which had weak similarity to papillomaviruses (48). Another study, by Casey et al. (17), identified a retrovirus in Hawaiian green sea turtles, but it is believed to be an endogenous retrovirus unrelated to FP. Therefore, the objective of this study was to use viral metagenomics to identify DNA viruses associated with FP in a Florida green sea turtle.

Viral particles were purified from a sea turtle fibropapilloma using a combination of size selection, density-dependent centrifugation, and nuclease treatment. DNA was extracted from the purified viruses, amplified with sequence-independent amplification, and shotgun sequenced. A novel single-stranded DNA virus with extremely limited similarity to known viruses was discovered. The genome of this virus, which we have named sea turtle tornovirus 1 (STTV1), was completely sequenced. In addition, several closely related variants of this virus were identified, indicating that STTV1 circulates as a quasispecies. Here we describe the STTV1 genome sequence, prevalence among green sea turtles, and variability within the STTV1 quasispecies.

MATERIALS AND METHODS

Sea turtle fibropapilloma sample. A wild juvenile green turtle with FP of category 3 severity (7) was captured from Lake Worth Lagoon, FL, in 2006 and transferred to the Clearwater Aquarium and later to the Mote Marine Aquarium for rehabilitation. Severe external fibropapillomas were present, and X-rays revealed the presence of internal fibromas. Due to its deteriorating condition, the turtle was humanely euthanized. External fibropapillomas from this turtle were biopsied and kept at −80°C until processed.

Viral purification and DNA extraction. The external fibropapilloma was homogenized in sterile SM buffer (50 mM Tris, 10 mM $MgSO_4$, 0.1 M NaCl, pH 7.5) using a mortar and pestle. Host cells were removed by centrifugation at 10,000 × *g* at 4°C for 10 min. The supernatant was then filtered through a 0.45-μm filter (Millipore, Billerica, MA) and loaded onto a cesium chloride step gradient consisting of 1 ml each of 1.7, 1.5, and 1.2 g/ml in sterile SM buffer (14). The gradient was ultracentrifuged at 61,000 × *g* at 4°C for 3 h, and the viral fraction between 1.2 g/ml and 1.5 g/ml was collected. The viral fraction was then incubated for 10 min with 0.2 volumes of chloroform. The supernatant containing the virus was removed from the chloroform and incubated with 2.5 U DNase I (Sigma-Aldrich, St. Louis, MO) at 37°C for 3 h. The DNase-treated sample was concentrated and washed twice with sterile SM buffer on a Microcon 30 column (Millipore). DNase activity was then inhibited by adding EDTA to a final concentration of 20 mM. Finally, DNA was extracted from the purified viruses using the QIAamp MinElute virus spin kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. To ensure the effectiveness of the viral purification procedure, the extracted DNA was assayed for turtle DNA contamination using eukaryotic rRNA gene PCR (universal primer A, 5′-AACCTGGTTGATCCTGCCAGT-3′; universal primer B, 5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (62) and for mitochondrial DNA contamination with conserved primers (L14841, 5′-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3′; H15149, 5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3′) (44).

Viral metagenome library construction and sequencing. Clone libraries were prepared for shotgun sequencing as described previously (14). First, viral DNA was amplified with sequence-independent amplification using the GenomiPhi V2 DNA amplification kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. This method uses isothermal strand displacement amplification with the phi29 DNA polymerase and random hexamers to amplify total genomic DNA by 2 to 3 orders of magnitude (10).

The amplified DNA was then randomly fragmented, linker adapted, and further amplified with primers to the linkers using the GenomePlex whole-genome amplification kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The reaction product was processed with a PCR cleanup kit (Mo Bio Laboratories, Solana Beach, CA) and then cloned into the pCR4 vector using a Topo TA cloning kit (Invitrogen, Carlsbad, CA). Transformants were screened for inserts and then sequenced by Agencourt Bioscience (Beverly, MA) using the M13 forward primer. A total of 276 transformants were sequenced, and the sequences were trimmed for vector and read quality using the Sequencher 4.7 software program (Gene Codes, Ann Arbor, MI). Trimmed metagenomic sequences (n = 211) were assembled into contigs (contiguous sequences) using a minimum overlap of 25 bp and a minimum match percentage of 85%. All assemblies were examined manually and verified experimentally with PCR.

Complete genome sequencing of sea turtle tornovirus 1 and its quasispecies variants. Both trimmed sequences and assembled contigs from the viral metagenome were analyzed using BLASTX and TBLASTX against the GenBank nonredundant database (4, 5). Several sequences with amino-acid-level similarity to chicken anemia virus (CAV) were noted, and efforts were focused on completing the genome sequence of this virus. PCR primers (Table 1) were designed to amplify and sequence the genomic regions between the contigs. Unless otherwise specified, all PCRs were in a final volume of 50 μl containing 200 μM deoxynucleoside triphosphates, 1 U of Red *Taq* polymerase (Sigma-Aldrich), 1× Red *Taq* reaction buffer, 1 μM of the specific primers, and either 5 μl of the sample DNA or 1 μl of sample DNA amplified by using Genomiphi. PCR with the primer set STTV1-b was performed to amplify a region of 1,571 nucleotides in order to confirm the assembly of the metagenomic sequences. PCR was performed using the STTV1-c inverse primers to confirm the circular nature of the STTV1 genome.

A series of amplicons with different sizes were observed from the STTV1-c PCR and gel electrophoresis, reflecting a hypervariable region (HVR) in the genome that was difficult to sequence and required a different sequencing strategy. Two strategies were employed to sequence the HVR. The first strategy included rolling-circle amplification (Templiphi; GE Healthcare) with random hexamer primers, followed by touchdown PCR using STTV1-c primers over a

gradient of annealing temperatures (Table 1), pooling of PCR products, and Topo TA cloning. The second strategy was identical to the first one with the exception that rolling-circle amplification was not used. For each tornovirus-positive turtle, 10 clones were selected and sequenced with the M13 forward and reverse primers. Each clone was sequenced at least three times. The HVR sequences were regarded as valid only if both ends of the HVR sequence aligned to the main STTV1 sequence with a total overlap of at least 80 nucleotides, excluding the primer region. Since the genomes were identical with the exception of the HVR, these were regarded as variants of the STTV1 quasispecies. Putative open reading frames (ORFs) in the genome were predicted using the NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf) with a minimum ORF size of 100 amino acids, and the genome was annotated using the SeqBuilder software program (DNAstar, Madison, WI). Repeats were analyzed using the Tandem Repeats Finder database (http://tandem.bu.edu/trf/trf.html) (9) and by aligning the sequence with itself using BLAST (http://www.proweb.org/proweb/Tools/selfblast.html). The potential nuclear localization signal was predicted using PREdictNLS (20).

Polarity and single-stranded nature of STTV1. The polarity of the STTV1 genome was determined using the strand-specific primer extension method (51). Purified virus DNA was amplified linearly for 50 cycles with either the STTV1-d sense primer or the STTV1-d antisense primer (Table 1) in a reaction mixture of 50 μl. The strand-specific primer extension was repeated with the same primer for an additional 50 cycles by adding the entire first reaction mixture to a new 50-μl PCR. Five microliters of the primer extension products were then used as the target in a PCR with the STTV1-e primers, which were internal to the strand-specific primers (Table 1). The PCR product intensities were compared by gel electrophoresis and ethidium bromide staining. The strand of packaged viral DNA is complementary to the strand-specific primer with a stronger PCR signal.

The single-stranded nature of the purified STTV1 DNA was determined by restriction enzyme digestion followed by PCR with primers flanking the restriction site. Two microliters of purified viral DNA was digested with 10 U of AluI (New England Biolabs, Ipswich, MA) for 60 min in the presence of 1× enzyme buffer in a total reaction of 20 μl. Five microliters of the digested product was amplified by PCR using STTV1-e Forward and STTV1-f Reverse (Table 1), which flanked the digestion site. Resistance to digestion and subsequent amplification by PCR confirmed the single-stranded nature of the packaged viral genome.

PCR detection of STTV1 prevalence in Florida green sea turtles. To investigate the prevalence and tissue specificity of the newly discovered STTV1, numerous sample types (fibropapillomas, various internal organs, blood, and external swabs) were examined using PCR with the STTV1-a primers (Table 1). Fibropapillomas from four captive green turtles were biopsied at Mote Marine Aquarium, FL, and total DNA was extracted from the fibropapillomas using the DNeasy Blood & Tissue kit (Qiagen, Valencia CA). The fibropapilloma from one of the turtles was positive for STTV1, and therefore, oral, conjunctival, cloacal, and unaffected skin swabs, as well as a blood sample from this turtle, were tested using the same extraction and PCR protocols.

This PCR-positive turtle was later euthanized due to deteriorating health conditions. To further examine the distribution of STTV1 in this turtle, external fibropapilloma, internal fibroma, blood, kidney, liver, lung, pancreas, spleen, stomach, heart, muscle, esophagus, and urinary bladder were biopsied, and DNA was extracted and tested by PCR with the STTV1-a primers. To determine the sensitivity of this assay, PCR was performed with the STTV1-a primers on a dilution series of positive control DNA. The tissues were also tested for FPTHV using PCR with FPTHV-specific primers (GTHV1, 5′-TGTCTGGAGGTGGC GGCCACG-3′; GTHV2, 5′-GACACGCAGGCCAAAAAGCGA-3′) (55). The FPTHV PCR conditions were 94°C for 5 min and 35 cycles of [94°C for 30s, 62°C for 30s, and 72°C for 30s], followed by 72°C for 5 min.

Results demonstrated that STTV1 could be detected in external swabs from the positive turtle; therefore, swabbing was used as a noninvasive sampling method to test a large number of FP and non-FP turtles for the presence of STTV1. Conjunctival swabs, oral swabs, fibropapilloma swabs, skin swabs, and/or blood samples were taken from a total of 17 captive and 45 wild green sea turtles. The 17 captive turtles were being kept at Mote Marine Aquarium and Clearwater Marine Aquarium in Florida, and 12 of these turtles were affected by FP. Twenty-six of the wild turtles were captured as part of a routine monitoring survey at the Indian River Lagoon (Melbourne Beach, FL), and 15 of these turtles had FP. Nineteen of the wild turtles were captured in Trident Submarine Basin (Cape Canaveral, FL), and none of these turtles had FP. In addition, leeches were collected opportunistically when wild green turtles were sampled to check for the presence of STTV1. DNA was extracted from the swabs and leeches using the DNeasy Blood & Tissue kit and tested by PCR with the STTV1-a primers (Table 1).

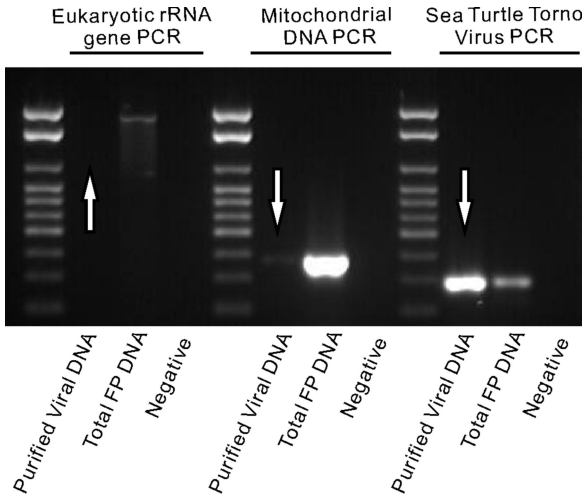

FIG. 1. Effectiveness of the virus purification procedure as determined by PCR assays. "Purified viral DNA," as indicated by arrows, was extracted from viral particles purified by filtration, density-dependent centrifugation, and nuclease treatment. "Total FP DNA" was extracted from a sea turtle fibropapilloma without any purification of viruses. The amount of host and mitochondrial DNA was greatly reduced in the purified viral DNA, while STTV1 was enriched in this fraction.

Nucleotide sequence accession numbers. The complete genome sequences for each of the nine STTV1 quasispecies variants have been deposited to GenBank with the accession numbers EU867816 to EU867824.

RESULTS

Purification of viruses from fibropapilloma tissue. Using a combination of size selection, density-dependent centrifugation, and nuclease treatment, viral particles were successfully purified from the sea turtle fibropapilloma. The purified viral fraction tested negative for turtle host DNA by PCR with the eukaryotic rRNA gene PCR (62) and was highly reduced in mitochondrial DNA as assessed by universal mitochondrium PCR (44) (Fig. 1). PCR with the STTV1-e primers revealed an enrichment of STTV1 in the purified viral fraction. This demonstrates the efficacy of the methods described here for purifying viral particles away from contaminating nucleic acids in animal tissues.

Analysis of viral metagenomic sequences and discovery of a novel sea turtle tornovirus. BLAST analysis of the 211 viral metagenomic sequences revealed 13 sequences (6%) that assembled into contigs with weak amino acid similarity to CAV, therefore indicating the discovery of a new virus (STTV1). These sequences were used to design PCR primers to complete the STTV1 genome sequence and verify the assembly of the metagenomic sequences.

Eleven sequences of the viral metagenome were similar to bacteriophages, which likely infect the turtle's normal bacteria flora. Some of the sequences had amino acid similarity to bacteria and *Plasmodium*, which may be the result of imperfect viral purification or may be viral sequences that have no viral homologs in the database. The majority of the remaining sequences had no similarities in GenBank.

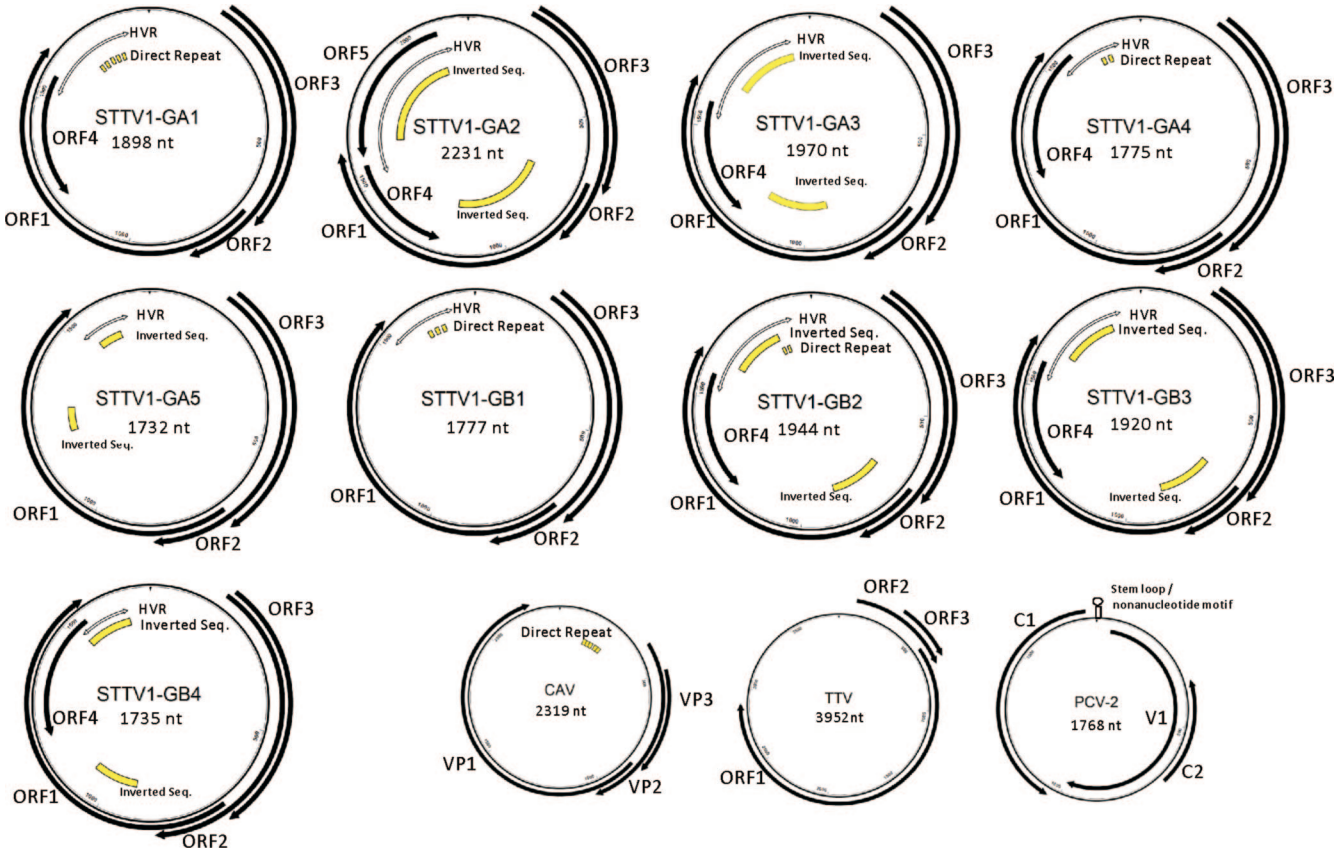

FIG. 2. Genome characteristics and organization of STTV1 variants. Variants STTV1-GA1 to -GA5 originated from a single green turtle. STTV1-GB1 to -GB4 originated from a second green turtle. CAV, TTV, and PCV-2 are included for comparison of genome organization. Genome annotation is based on sequence data only, since there are no transcriptional data available for STTV1. CAV, TTV, and PCV-2 genome organization has been redrawn based on GenBank annotation and as depicted in the work of Todd (63). Note the similarity in genome organization between STTV1, CAV, and TTV, which is distinct from the two large opposite ORFs of PCV-2.

Screening of fibropapillomas for STTV1 by PCR. Including the turtle where STTV1 was originally discovered, two out of five fibropapilloma samples tested positive for STTV1. Both STTV1-positive turtles were severely afflicted by FP and had developed extensive fibropapillomas and internal fibromas when the samples were taken. The sensitivity of the STTV-1a PCR was determined to be approximately 10 targets.

Prevalence and tissue specificity of STTV1. In one of the STTV1-positive turtles, STTV1 was detected in external fibropapilloma, internal fibroma, blood, kidney, liver, lung, pan-

TABLE 2. Genome characteristics of STTV1 variants

Quasispecies variant	Use of rolling circle amplification in sequencing of HVR[a]	Genome length[b] (nt)	ORF1 length (nt)	No. of CCATCAGAGAATC direct perfect repeats	Incorporation of inverted sequence from non-HVR		Presence of ORF4 (length, nt)	Presence of ORF5	Poly(A) signal (position relative to ORF1 stop codon)[c]
					Position of non-HVR being incorporated	Position of inverted sequence in HVR			
STTV1-GA1	No	1,898	933	5			Yes (360)	No	Yes (−22)
STTV1-GA2	Yes	2,231	933	0	692–1163	1655–2126	Yes (360)	Yes	Yes (−22)
STTV1-GA3	Yes	1,970	933	0	914–1163	1655–1904	Yes (360)	No	Yes (−22)
STTV1-GA4	Yes	1,775	840	2			Yes (360)	No	Yes (64)
STTV1-GA5	Yes	1,732	855	0	1220–1300	1551–1631	No	No	No
STTV1-GB1	No	1,777	858	3			No	No	Yes (47)
STTV1-GB2	Yes	1,944	933	2	692–886	1622–1816	Yes (360)	No	Yes (−22)
STTV1-GB3	Yes	1,920	933	1	692–886	1622–1816	Yes (360)	No	Yes (−22)
STTV1-GB4	Yes	1,735	882	0	914–1062	1521–1669	Yes (339)	No	No

[a] Two sequencing strategies were employed, as described in Methods.
[b] Genome length was determined by aligning the HVR sequence into the STTV1 non-HVR sequence of the same turtle to complete the circular genome.
[c] The number of nucleotides downstream of the stop codon is shown as a positive integer, and that upstream is shown as a negative integer.

creas, spleen, stomach, heart, esophagus, and urinary bladder samples. STTV1 was not detected in the muscle sample or in an internal fibroma biopsied from the right lung. STTV1 was also found in external swabs collected from the conjunctiva, oral cavity, cloaca, external fibropapilloma, and unaffected skin. In contrast, FPTHV was detected only in the fibropapillomas and fibromas and was not found anywhere else in the turtle. Since STTV1 could be detected in external swabs collected noninvasively, this method was used to screen more captive and wild green sea turtles for STTV1.

A total of 62 green turtles (27 with FP and 35 without FP) were swabbed and tested for STTV1. The presence of FP was determined by careful inspection for fibropapillomas (7). The only turtles positive for STTV1 were the same two turtles identified previously by screening of fibropapillomas. All other turtles were negative for STTV1 in the conjunctival swabs, oral swabs, fibropapilloma swabs, skin swabs, and/or blood samples. Interestingly, STTV1 was detected in leeches collected from one of the wild green sea turtles captured in the Indian River Lagoon (Florida), suggesting that leeches may be a possible vector for the transmission of STTV1.

Sequence analysis of the sea turtle tornovirus genome and its quasispecies variants. The STTV1 genomes from the two positive turtles were completely sequenced. In order to complete the STTV1 genome, it was necessary to sequence the conserved region of the genome (non-HVR) and the HVR separately. The non-HVR was amplified with the STTV1-b primers. Over the 1,571-nucleotide (nt) STTV1-b amplicon, the viruses from the two positive turtles differ at only 10 positions (sequence divergence of 0.6%); thus, the non-HVR of STTV1 were almost identical.

PCR using the STTV1-c inverse primer set confirmed the circular nature of the STTV1 genome. PCR with these primers also identified a HVR, in which nine different PCR amplicons were identified in the two turtles. The HVR varied in length by almost 500 nucleotides, leading to quasispecies genome sizes ranging from 1,732 to 2,231 nucleotides (Fig. 2 and Table 2). Five variants, named STTV1-GA1 to STTV1-GA5, originated from the same turtle, while the other four variants (STTV1-GB1 to STTV1-GB4) were from the second turtle. The sequence alignment of the HVR of the nine variants is shown in Fig. 3a. At both ends of the HVR (positions 1507 to 1629 and positions 2125 to 2166), variation is due only to insertions or deletions. In the middle of the HVR (positions 1630 to 2124), variations include nucleotide insertions, deletions, and substitutions. Some of the variations were shared between two or more variants.

Sequence analysis demonstrated that six of the variants incorporated an inverted sequence from the non-HVR region into the middle of the HVR (Fig. 2 and Table 2). For example, STTV1-GB2 and STTV1-GB3 incorporated identical sequences from the non-HVR into the HVR. The HVR of STTV1-GA2, STTV1-GA3, and STTV1-GB4 shared another inverted non-HVR sequence, but the length of the incorporated sequence was variable. The HVR of STTV1-GA5 contained an inverted non-HVR sequence that was not found in the other variants in this study. The sequences incorporated into the HVR were all located inside the sequence annotated as ORF1.

ORF1 was present in all nine variants, and it spanned both the conserved regions and HVR. Due to the addition and deletion of nucleotides in the HVR, the length of ORF1 varied from 840 to 933 nt (Table 2). ORF1 was characterized by an arginine-rich region, containing 23 arginines in the first 43 amino acids (53%). The arginine-rich region of ORF1 was similar to the arginine-rich region in other single-stranded DNA viruses, such as anelloviruses, gyroviruses, and circoviruses. The ORF1 arginine-rich region was located in the non-HVR region and therefore was shared by all STTV1 genome variants. Analysis using a nuclear localization signal prediction tool revealed that the arginine-rich region in ORF1 had strong potential as a nuclear localization signal. Other than the characteristic of an arginine-rich region, the remaining sequence of the STTV1 ORF1 had no amino acid level similarity to any proteins in the nonredundant database.

Unlike ORF1, both ORF2 and ORF3 were located entirely within the non-HVR region. ORF2 was 687 nt in length, and it shared low (25%) amino acid identity with the CAV VP2 protein over a region of 217 amino acids. STTV1 ORF2 had 16 amino acids identical to those of the CAV pfam (04861) over a region of 40 amino acids. STTV1 ORF3 was 522 nt in length, and it had no similarity to any known proteins.

While ORF1, ORF2, and ORF3 were found in every genome variant sequenced, two other putative ORFs were found only in some of the variants, depending on the sequence of the HVR (Fig. 2 and Table 2). ORF4, which had no similarity to any known proteins, was present in seven of the nine variants. Although the start codon of ORF4 was in the HVR, the length of ORF4 was typically 360 nt, except in STTV1-GB4, where the length was 339 nt.

ORF5 was present only in STTV1-GA2, where it was 522 nt in length. Sequence analysis showed that the first 471 nt of this ORF were the inverted repeat of a non-HVR sequence near the beginning of ORF1 (Fig. 2 and Table 2). Therefore, ORF5 resembled ORF1 for the most part, including the arginine-rich region. ORF5 was shorter than ORF1 and had no similarity to any known protein except the arginine-rich characteristic.

A TATA box (TATAAA) was identified in the non-HVR sequence, and thus, all of the variants contain a TATA box in the genome. The TATA box was arbitrarily designated to be the first nucleotide of the circular genome, and it was located 171 nt upstream of the start codon of ORF2. Additionally, a poly(A) signal (AATAAA) was identified in the HVR. The poly(A) signal was positioned 22 nt upstream of the stop codon of ORF1 in five of the variants (STTV1-GA1, -GA2, -GA3, -GB2, and -GB3 in Table 2), while it was 47 nt and 64 nt downstream for two variants (STTV1-GB1 and -GA4). The remaining two variants (STTV1-GA5 and -GB4) had no poly(A) signal.

Direct repeats were recognized in the HVR sequences of four variants (Fig. 3b). Two types of direct repeats were recognized: a perfect direct repeat of 13 nt in length (CCATCA GAGAATC) and a near-perfect direct repeat of 11 nt in the form of (T/C)CATCAGAGAATC. The 13-nt perfect direct repeats occurred between two to five times in different variants and were interspaced with the 11-nt near-perfect direct repeat and/or gaps of other short sequences.

Genomic features distinguishing STTV1 quasispecies variants. Nucleotide insertions, deletions, and substitutions in the HVR contribute to the array of genomic features seen in the

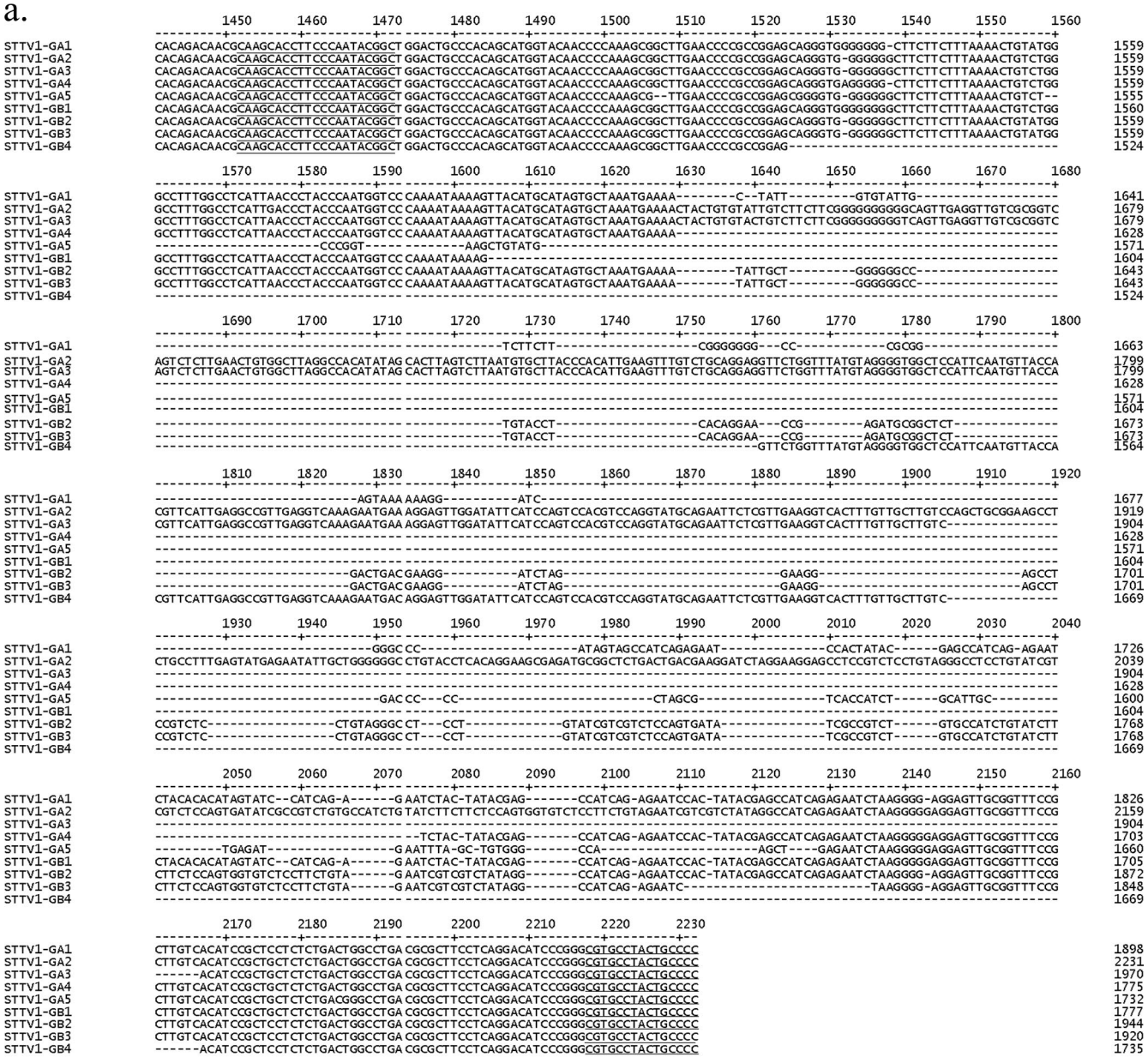

FIG. 3. (a) Alignment of the nine HVR sequences. The variants STTV1-GA1 to STTV1-GA5 originated from a single green turtle, while STTV1-GB1 to STTV1-GB4 originated from a second turtle. Primer STTV1-B Forward (5′ CAAGCACCTTCCCAATACGGC 3′) is underlined. Part of STTV1-B Reverse (5′TTATAGGGGCAGTAGGCACG 3′) is underlined, but the first 5 nt of the primer were located at positions 1 to 5 in the circular genome and are not shown in the alignment. (b) Direct repeats in the HVR of the variants STTV1-GA1, STTV1-GA4, STTV1-GB1, and STTV-GB2. PR, 13-nt perfect direct repeat in the form of CCATCAGAGAATC; NPR, 11-nt near-perfect direct repeat in the form of (T/C)CATCAGAGAATC. The position of the last nucleotide shown in each variant is noted on the right.

nine different STTV1 quasispecies variants (Fig. 2 and 3a; Table 2). First, differences in the length of the HVR contribute to variation in the STTV1 genome size, which ranges from 1,735 to 2,231 nt. Second, the HVR sequence generates a different amino acid composition and length of ORF1, which spans between the HVR and non-HVR in each of the variants. The length of ORF1 can vary from 840 to 933 nt. Third, ORF4 is found in seven of the nine variants, while ORF5 is found in one variant. Fourth, the number and pattern of direct perfect repeats and direct near-perfect repeats differ in the HVR of various variants (Fig. 3b). Fifth, various lengths of non-HVR sequences are incorporated into the HVR in inverted orientation in some of the variants (Fig. 2 and Table 2). Sixth, a poly(A) signal was found in most variants upstream or downstream of the stop codon of ORF1.

The combination of these features makes up the different genome characteristics of each variant (Fig. 2 and Table 2). STTV1-GA1 had a genome of 1,898 nt, with a GC content of 51.8%. It contained putative ORF4 and five 13-nt perfect repeats interspaced with three 11-nt near-perfect repeats. The STTV1-GA2 genome was 2,231 nt in length and was characterized by the presence of ORF4 and ORF5. It incorporated an inverted sequence from the non-HVR, making it the largest STTV1 variant sequenced. No direct repeat was found in the STTV1-GA2 genome. STTV1-GA3 (1,970 nt), STTV1-GB3 (1,920 nt), and STTV1-GB4 (1,735 nt) all contained ORF4 and inverted sequence from the non-HVR but had no or only one copy of the direct repeat sequence. STTV1-GA4 (1,775 nt) contained ORF4, as well as two 13-nt perfect repeats interspaced with two 11-nt near-perfect repeats. STTV1-GA5 (1,732 nt) had an inverted sequence from the non-HVR but lacked ORF4. STTV1-GB1 (1,777 nt) had three 13-nt perfect repeats interspaced with two 11-nt near-perfect repeats and also lacked ORF4. The STTV1-GB2 genome (1,944 nt) contained ORF4, incorporated an inverted sequence from the non-HVR, and had two 13-nt perfect direct repeats interspaced with one copy of the 11-nt near-perfect direct repeat sequence.

Polarity and strandedness of the purified STTV1. The STTV1 DNA packaged in the viral particle was single stranded, since the purified STTV1 DNA treated with a restriction enzyme could subsequently be amplified by PCR over the restriction enzyme recognition site.

ORF1, ORF2, and ORF3 were found consistently in every STTV1 variant, and since these three ORFs were likely to be transcribed, they were considered positive sense. Polarity experiments demonstrated that the viral particle contains a negative-sense genome, which is the reverse complement of the sequence shown in this article.

DISCUSSION

This article demonstrates the use of viral metagenomics to discover novel viruses directly from an animal tissue. We have successfully purified virus particles against a high background of host DNA (Fig. 1) and have identified and completely sequenced the genome of a novel sea turtle virus, STTV1, from the purified DNA.

STTV1 possesses a single-stranded circular genome, and most of its genome has no similarities to any known viruses. ORF2 has weak (25%) amino acid level similarity to the VP2 protein of CAV. The remainder of the genome has no amino acid similarity to any protein, except that the arginine-rich region of ORF1 and ORF5 is similar to those of anelloviruses, gyroviruses, and circoviruses. The STTV1 genome is so unique that it is highly unlikely to have been discovered by degenerate primers or panviral microarrays based on known viruses. Based on its genome sequence, we believe that STTV1 may represent a new viral genus of the *Circoviridae* family or possibly even a new viral family.

The genome organization of STTV1 is more similar to that of CAV (the only member of the *Gyrovirus* genus) and *Torque teno virus* (TTV) (a member of the floating *Anellovirus* genus) than to those of circoviruses (Fig. 2). The STTV1 genome consists of a region of direct repeats followed by short ORF2 and ORF3 sequences, and ORF1 starts near the end of ORF3 (Fig. 2). CAV is characterized by a similar genome organization pattern: repeat/promoter region at the noncoding part of the genome, followed by VP2 and VP3, with VP1 starting immediately after VP3 (Fig. 2). Likewise, genome annotation of TTV using sequence data reveals a similar ORF organization: short ORF2 and ORF3, followed by a long ORF1 (63). In contrast, circoviruses have a different genome organization from STTV1, containing two long ORFs (V1 and C1) located on opposite strands (Fig. 2) (63). STTV1 also lacks the conserved stem-loop structure and the nonanucleotide motif of circoviruses (64). It should be noted that transcriptional studies have recently updated the ORF annotations of CAV and TTV (41, 42), but the discussion here concerns only annotation based on sequence data, since there is no transcriptional data from STTV1 to compare with at this time. There are currently two families of single-stranded DNA (ssDNA) animal viruses, namely, *Circoviridae* and *Parvoviridae*, as well as the floating anellovirus genus. The discovery of STTV1 provides a link between anelloviruses and gyroviruses (*Circoviridae*), since STTV1 shares some genomic features with both groups of viruses. STTV1 may therefore be an important evolutionary intermediate that can enhance our understanding of the evolution of ssDNA viruses.

ORF1, ORF2, and ORF3 are found consistently in every STTV1 variant (Fig. 2) and are located on the opposite strand from the strand packaged in viral particles, suggesting a negative-sense genome organization. ORF4 and ORF5 are found only in some variants, and they are on the opposite strand of ORF1 to ORF3. Further studies are needed to determine if all the ORFs are transcribed, which will reveal whether STTV1 is a negative-sense or ambisense virus. Comparatively, TTV and CAV also have three ORFs that are arranged in negative-sense organization, while members of the *Circovirus* genus have an ambisense genome organization (Fig. 2).

STTV1 ORF1 has an arginine-rich region similar to that in other ssDNA viruses, such as anelloviruses, gyroviruses, and circoviruses (49, 50). STTV1 ORF1 contains 23 arginine residues in the first 43 amino acids (53%). In comparison, ORF1 of the anellovirus TTV contains 44 arginines over the first 82 amino acids (54%), VP1 of the gyrovirus CAV contains 23 arginines over the first 53 amino acids (43%), and ORF C1 of porcine circovirus type 2 (PCV-2) contains 17 arginines over the first 40 amino acids (43%). Interestingly, an arginine-rich region can be identified in almost all of the anelloviruses,

gyroviruses, and circoviruses, but it is not normally found in other ssDNA viruses, such as parvoviruses, geminiviruses, inoviruses, nanoviruses, and microviruses. The arginine-rich region in ORF1 of the STTV1 genome has strong potential as a nuclear localization signal. For herpes simplex virus type 1 (38) and PCV-2 (46), an arginine-rich region is responsible for nuclear localization. The arginine-rich motif in TTV was originally suggested to be a nuclear localization signal, but subsequent expression studies have not confirmed this prediction (53).

In the affected turtles, numerous STTV1 genome variants are present at any given time, demonstrating that STTV1 circulates as quasispecies. The variants are identical over the majority (>80%) of their genome, with the exception of an extremely divergent HVR. Cloning and sequencing of the HVR using STTV1-c PCR revealed that all of the cloned sequences are unique. Therefore, the STTV1 genome variants have not been sequenced exhaustively yet, and the present study shows only representative variants of the quasispecies. Additional sequencing effort will likely reveal more STTV1 variants with different HVR sequences.

The STTV1 quasispecies contain high sequence diversity, which can be the result of multiple infections or of having descended from a single variant. If the latter is true, this level of divergence and rate of mutation are consistent with those of other small ssDNA viruses (27). Similarly high sequence diversity is present among other ssDNA viruses, including anelloviruses, gyroviruses, geminiviruses, and parvoviruses (26, 33, 49, 58–60, 65). Recent studies have shown that these ssDNA viruses can reach mutation rates comparable to those of RNA viruses, whose polymerase lacks proofreading ability (27). The high level of sequence divergence among the STTV1 quasispecies supports the theory that evolutionary rates in viruses are controlled not only by polymerase fidelity but also by genomic architecture and replication speed (27). It will be intriguing to investigate how the STTV1 quasispecies generates such high levels of sequence divergence. The function of ORF1 is unknown, but the arginine-rich region and analogy to the genome organization of CAV suggest that it may be the capsid protein. In a culture-based study of CAV (58), a HVR in the capsid protein (VP1) led to CAV genome variants with increased rates of replication or spread of infection. Since ORF1 of STTV1 also includes a HVR, further studies should investigate the effects of this sequence divergence on virus replication.

Of the 62 turtles (27 with FP and 35 without FP) examined in this study, STTV1 was present in only two of the turtles. Both STTV1-positive turtles had severe FP, including both external fibropapillomas and internal fibromas. One of the STTV1-positive turtles also had regrowth of fibropapillomas after surgical removal. Since STTV1 was not found in the majority of the FP turtles, it does not appear to be the causative agent of FP. However, STTV1 may be involved in a coinfection with the FPTHV in some terminal cases, since both affected turtles were positive for FPTHV and STTV1. Interestingly, while FPTHV was found only in fibropapilloma tissue, affected turtles were systemically infected with STTV1, which could be detected in the fibropapilloma and in the blood, conjunctiva, oral cavity, unaffected skin, and many internal organs. The role of STTV1 in sea turtles is currently unknown. It is possible that STTV1 is a commensal virus in sea turtles, that STTV1 negatively impacts the sea turtle's immune system (similar to CAV in chickens [1]), or that STTV1 is an opportunistic pathogen in these turtles since they were severely afflicted with FP. Future studies will be needed to examine the role of STTV1 in FP progression or immune status.

Virus purification and shotgun sequencing (viral metagenomics) directly from tissues have a number of advantages over other virus discovery methods. Since viral metagenomics does not rely upon similarity to known viral genomes, this method can be used to describe a whole new spectrum of viral genomes that could not be identified through degenerate PCR or panviral microarrays (66). Compared to studies in which viruses were replicated in cell culture prior to shotgun sequencing (39, 43), viral metagenomics performed directly with animal tissue samples circumvents the limitation that many viruses cannot be grown in cell culture or that appropriate cell lines are unavailable. Direct metagenomic sequencing without the initial virus purification step can be useful in identifying potential viral pathogens (such as those in references 21, 30, and 52); however, viral sequences represented less than 0.05% of the entire sequencing effort in these studies, compared to the 6% viral sequence recovery rate in this study. In addition, digital transcript subtraction, a downstream application for subtracting host sequence after direct metagenomic sequencing (30), will not be applicable for virus discovery in the majority of animals for which a genome sequence is not available. Compared to rolling-circle amplification, which selects for viruses with circular genomes (56, 57), the method described here can be used to detect viruses with both linear and circular genomes. Representational difference analysis discovers viruses utilizing DNA hybridization between tester nucleic acids from affected tissue and driver nucleic acids from uninfected tissue, but it requires a large amount of both infected and unaffected materials (23), which are not always available, especially for humans, endangered species, or animals involved in unexpected mortality events.

Viral metagenomics is a promising technique for virus discovery, enabling the identification of viruses with limited homology to known viruses. For example, the STTV1 genome described here is extremely different from genomes of any previously described viruses. Currently, most known single-stranded DNA animal viruses have been identified in samples from birds and mammals using PCR with degenerate primers. The use of degenerate primers for viral discovery tends to be most successful with viruses from closely related hosts, limiting the potential for discovery of novel viruses in other classes of animals. STTV1 is only the second single-stranded DNA virus known in reptiles (the other is snake parvovirus [29]), and it is clearly different from other known ssDNA viruses. Since viral metagenomics can be used to discover viruses from many tissue types from a wide range of animals, this approach has the potential to significantly enhance the fields of virus discovery and infectious disease monitoring.

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