LETTER

Discovery of a novel potexvirus in the seagrass *Thalassia testudinum* from Tampa Bay, Florida

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Scientific Significance Statement

Seagrass meadows, the most widespread coastal ecosystem on the planet, fulfill numerous essential ecosystem functions. Given global declines in seagrass coverage and increasing urbanization of coastal areas, it is critical to better understand seagrass ecology, including the role of their associated microbiomes in productivity, resilience, and health. Although previous studies have investigated bacteria, fungi, and protists associated with seagrasses, information on viruses is currently lacking. In this study, we discovered a new single-stranded ribonucleic acid virus named turtlegrass virus X in the widespread subtropical species *Thalassia testudinum* (turtlegrass) in Tampa Bay, Florida. To our knowledge, this is the first report of a virus associated with this species of seagrass.

Abstract

Seagrass meadows are important coastal ecosystems that are declining worldwide. Given the profound impact of the microbiome on plant health, exploration of the seagrass microbiome is critical for proper ecosystem management and conservation. Although prior studies have investigated seagrass-associated bacteria, fungi, and protists, virtually nothing is known about viruses infecting these flowering marine plants. Here, we report genomic and microscopic evidence of a new positive-sense, single-stranded ribonucleic acid virus infecting apparently healthy *Thalassia testudinum* in Florida. The virus, named turtlegrass virus X (TVX), shares 66% genome-wide pairwise identity with foxtail mosaic virus, a potexvirus that infects terrestrial grasses. Quantitative polymerase chain reaction revealed TVX presence in 10% to 80% of *T. testudinum* leaves from two Tampa Bay sampling locations in February and August 2017, with an average viral load of 4.65×10^8 copies per mg leaf tissue. The discovery of TVX advances seagrass microbiome research, prompting further studies to assess its ecological impact.

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Seagrass meadows are among the most productive and widespread coastal ecosystems on the planet (Larkum et al. 2006). Seagrasses are a paraphyletic group of fully submerged marine angiosperms that convergently adapted to the marine environment between 70 and 100 million years ago (Les et al. 1997). There are only approximately 60 species of seagrasses compared to the 250,000 species of terrestrial angiosperms, reflecting the strict selective pressure posed by re-entry to the marine environment (Orth et al. 2006). As aquatic angiosperms, seagrasses fulfill numerous essential ecosystem functions, such as habitat stabilization, maintaining water quality, nutrient cycling, carbon sequestration, and supporting commercial fisheries by serving as a nursery site and food source for many marine species (Waycott et al. 2009). In addition to these functions, seagrass ecosystems can reduce exposure to bacterial pathogens of fishes, invertebrates, and humans (Lamb et al. 2017). Unfortunately, seagrasses are currently disappearing at a rate of 110 km^2 yr^{-1} since 1980 due to various stressors, including eutrophication, coastal development, dredging activities, global climate change, and disease (Duarte et al. 2004; Orth et al. 2006; Waycott et al. 2009).

Recently, there has been a growing appreciation of the importance of microbial communities living in and on seagrasses, referred to as the seagrass microbiome (Bengtsson et al. 2017; Ettinger et al. 2017; Fahimipour et al. 2017; Ugarelli et al. 2017). Microbiomes, which include all microbes (archaea, bacteria, fungi, protists, and viruses) associated with a given organism, are key determinants of a plant's health and productivity by influencing processes such as nutrient uptake and immunity or by driving the evolution of the host (Turner et al. 2013; Rosenberg and Zilber-Rosenberg 2016). Most seagrass microbiome studies have focused on archaea, bacteria, and fungi; however, viruses have not been included in these efforts, and their impact on seagrass health remains unknown. Evidence for viral infection of seagrasses is extremely scarce, with only a single study describing a double-stranded (ds) ribonucleic acid (RNA) virus from the genus *Endornavirus* (family: *Endornaviridae*) infecting eelgrass (*Zostera marina*; Fukuhara et al. 2006).

Due to the importance of seagrasses in marine ecosystem dynamics and the lack of information regarding viral infection in this major group of marine autotrophs, our study sought to identify viruses in the subtropical seagrass *Thalassia testudinum* Banks ex König, also known as turtlegrass. *T. testudinum* is the climax seagrass species throughout the Caribbean (Williams 1990), and decadal changes in abundance and massive die-off events have been documented in Florida during the past 30 yrs (Durako 1994; Hall et al. 2016). Characterizing viruses associated with this ecologically important seagrass species will further advance seagrass microbiome research and knowledge regarding the role of viral infection in seagrass ecology.

Materials and methods

Virus discovery

Sample collection and purification of virus-like particles

Ten young seagrass leaves were sampled in December 2016 in the Terra Ceia Aquatic Preserve in Tampa Bay, Florida, USA (sampling point N01; Fig. 1; Supporting Information Table S1). Leaves were rinsed in situ with seawater and placed on ice in individual Ziplock© bags. In the lab, leaves were rinsed three times with MilliQ water and epiphytes were removed using sterile razor blades.

Virus-like particles (VLPs) were purified from the seagrass leaves following previous viral metagenomics methods (Ng et al. 2011). Approximately 100–150 mg of leaf tissue was placed in 2-mm ZR BashingBeads impact-resistant 2 mL tubes (Zymo Research) with 800 μL of suspension medium (SM) buffer (100 mmol L^{-1} NaCl, 8 mmol L^{-1} MgSO$_4$·7H$_2$O, 50 mmol L^{-1} Tris–HCl, pH 7.5). Samples were homogenized using a bead-beater for ~ 90 s and 800 μL of SM buffer was added to the homogenate.

After vortexing, tubes were centrifuged at 4°C for 10 min at 10,000 × g, and the supernatants were collected and filtered through 0.22 μm Sterivex filters (Millipore). Chloroform (20% final concentration) was added to 480 μL of the filtrate, vortexed vigorously, and incubated at room temperature for 10 min. After centrifuging for 30 s at 16,100 × g, 170 μL of the top aqueous layer was recovered. To remove unencapsidated nucleic acids, samples were treated with a nuclease cocktail (1 X TURBO DNase Buffer [Invitrogen], 21 U TURBO DNase [Invitrogen], 112.5 U Benzonase [Millipore], 400 U RNase I [Thermo Fisher Scientific], 4.5 U Baseline-ZERO™ DNase [Epicentre]) and incubated at 37°C for 2 h (Victoria et al. 2009). To inactivate the nucleases, a 20 mmol L^{-1} final concentration of ethylenediaminetetraacetic acid (pH = 8.0) was added to each reaction.

Nucleic acid extraction and sequence-independent single-primer amplification

Deoxyribonucleic acid (DNA) and RNA were extracted simultaneously from the purified VLPs, and a negative control composed of SM buffer alone, using the QIAamp MinElute Virus Spin Kit (Qiagen). Using sequence-independent single-primer amplification (SISPA)-primer "A" (5′-GTTTCCCAGT-CACGATANNNNNNNN-3′; Gaynor et al. 2007), cDNA was prepared using the SuperScript III First-Strand Synthesis System for real-time polymerase chain reaction (PCR) (Invitrogen) according to the manufacturer's instructions. Second-strand cDNA synthesis was performed with the Klenow Fragment DNA Polymerase (New England Biolabs). Five microliters of cDNA were subsequently amplified by PCR using SISPA primer "B" (5′ GTTTCCCAGTCACGATA; Gaynor et al. 2007). Amplification was performed using an initial denaturation of 95°C for 5 min followed by 5 cycles of 95°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min, then 40 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 1.5 min. A final extension at 72°C was
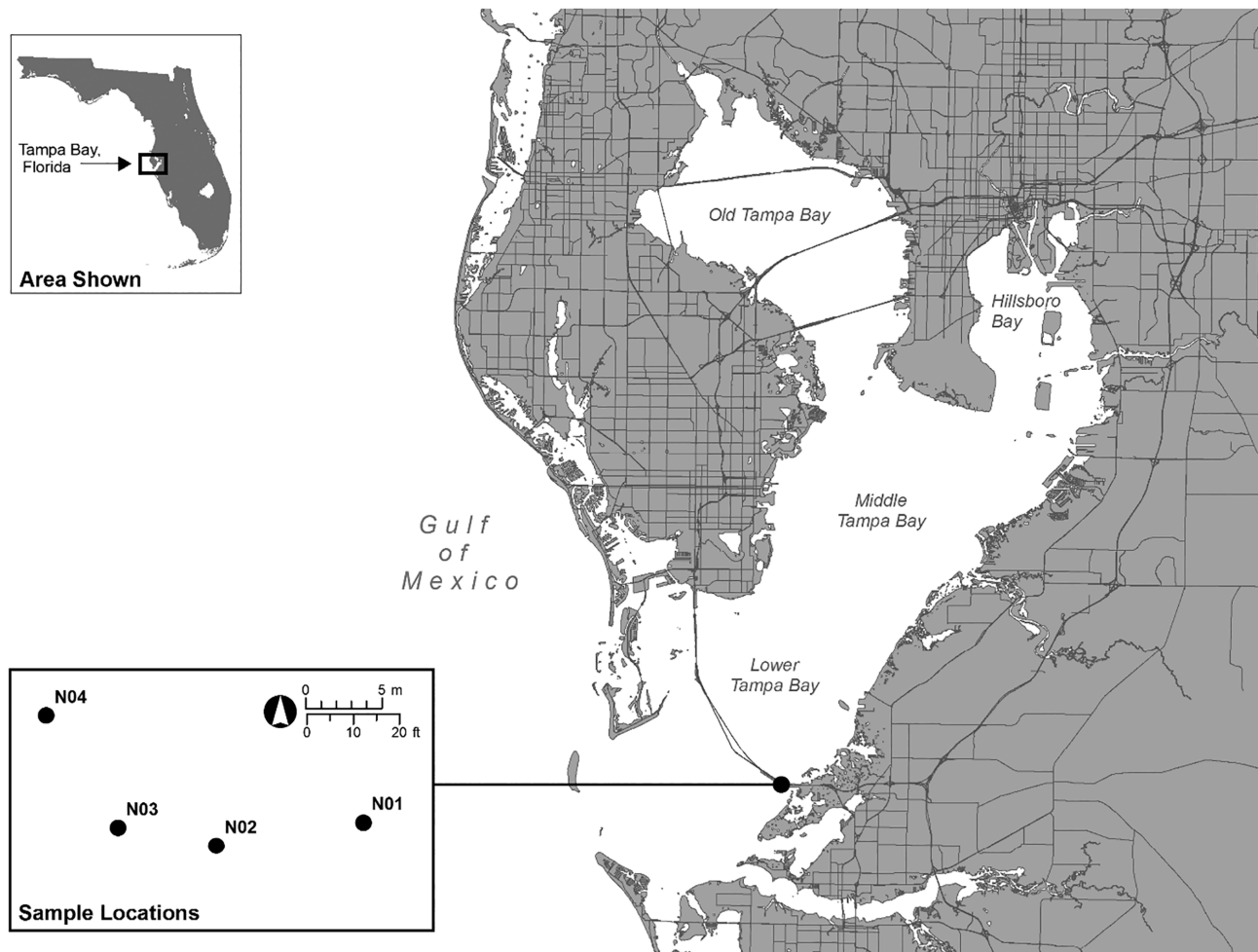
Fig. 1. Map showing the sampling location in the Terra Ceia Aquatic Preserve in Tampa Bay, Florida. In December 2016, samples were only collected from location N01 for SISPA. In February and August 2017, all four locations (N01–N04) were sampled to evaluate viral load and prevalence in seagrass leaves.

performed for 10 min. SISPA products were cloned using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific) and 96 cloned products were commercially sequenced by TACGen using the vector primers.

Sequence analysis and genome completion

Sequencher 4.7™ (Gene Codes) was used to quality filter and assemble sequences. Sequences and contigs were compared against the GenBank nonredundant database using BLASTx, revealing several sequences with up to 64% amino acid similarity to foxtail mosaic virus (FoMV; Bancroft et al. 1991). These sequences were then aligned against the most closely related FoMV genome (GenBank accession number AY121833) to enable completion of the genome through a combination of primer walking (primers designed using Primer 3, http://bioinfo.ut.ee/primer3/) and rapid amplification of cDNA ends (RACE; Scotto-Lavino et al. 2006) using Herculase II Fusion DNA polymerase (Agilent) with the primers and conditions described in Supporting Information Table S2. All cDNA templates, including

RACE reactions, were produced using the SuperScript IV First-Strand Synthesis System (Invitrogen) according to manufacturer's protocols. PCR products larger than 1 kb were cloned using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). All PCR products and clones were commercially sequenced by TACGen. All sequences were then assembled using the Geneious software package (Biomatters), resulting in the complete genome of a novel seagrass potexvirus, named turtlegrass virus X (TVX; GenBank accession number MH077559).

Prevalence and viral load

To determine geographic distribution, prevalence, and viral load of TVX, additional seagrass samples were collected from Tampa Bay and Florida Bay. In February and August 2017, 10 individual seagrass leaves were collected at sampling points N01, N02, N03, and N04 (Fig. 1), resulting in a total of 40 samples in each month. In May 2017, five seagrass leaves were taken from each of seven sites over a 50 m transect in Florida Bay (global positioning system locations in Supporting
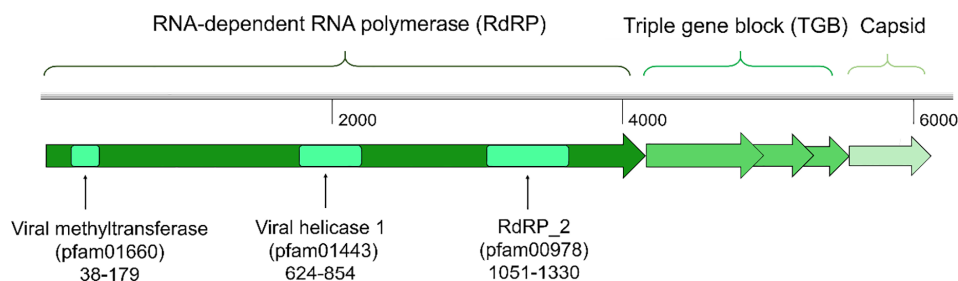
Fig. 2. Schematic representation of the genome organization of TVX. Three conserved domains found within the RdRP are shown, including viral methyltransferase, viral superfamily 1 RNA helicase, and RdRP_2 domains. Numbers under each domain refer to the RdRP amino acid positions.

Information Table S1). After cleaning the individual seagrass leaves as previously described, five punches per individual leaf were taken using sterile 5-mm-diameter biopsy punches (Sklar Instruments) corresponding to a total weight of approximately 50 mg per leaf. RNA was extracted from these five pooled punches using the RNA MiniPrep kit (Zymo Research). Quantitative PCR (qPCR) targeting the RNA-dependent RNA polymerase (RdRP) gene was used to quantify the prevalence and viral load of TVX. After RNA extraction, cDNA was synthesized using the SuperScript IV First Strand Synthesis System (Invitrogen) with random hexamer primers. Five microliters of 1 : 10 diluted cDNA was used for qPCR.

A commercially synthesized plasmid (IDT Technologies) containing a 200 nt sequence of the TVX RdRp gene (genome positions 132–332) was serially diluted from 10^7 to 10^2 copies per 5 μL using nuclease-free water to determine the sensitivity of the qPCR assay before testing the seagrass samples. The 25 μL qPCR mixture contained 5 μL of target cDNA, 12.5 μL of the TaqMan® Environmental Master Mix 2.0 (Life Technologies), 0.5 μmol L^{-1} of each primer (Supporting Information Table S2), and 0.25 μmol L^{-1} probe (Supporting Information Table S2). The qPCR mixture was incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was tested in duplicate, and each qPCR assay contained two negative (no template) control samples, for which nuclease-free water was used instead of cDNA. All standard curves had amplification efficiencies between 90% and 110% and $R^2 > 0.98$. Mean TVX amplicon copies in standard plasmid dilution series and cDNA samples were calculated through linear regression using the ABI 7500 Software v2.0.6 (Thermo Fisher Scientific) with default settings. As dsDNA was used to create the standard curve, values were divided by two to calculate mean copies of the single-stranded (ss) TVX cDNA. TVX concentrations in seagrass leaves were then back calculated to account for all dilutions from RNA extraction to cDNA synthesis and amplification. Statistica 64 (IBCO Statistica) was used for statistical analyses of qPCR results.

Phylogenetic analysis of viral sequences
The predicted RdRP amino acid sequences of 29 potexviruses and other closely related members of the family *Alphaflexiviridae* were aligned using the MUSCLE algorithm as implemented in MEGA 7 (Kumar et al. 2016). Aligned sequences were used to derive a neighbor joining phylogenetic tree using the Jones–Taylor–Thornton substitution model with pairwise deletion of gaps. Statistical support for each clade was assessed through bootstrap analysis with 1000 replicates. The Sequence Demarcation Tool Version 1.2 (Muhire et al. 2014) was used to evaluate the range of genome-wide pairwise identities for members of the genus *Potexvirus*.

Transmission electron microscopy
VLPs for transmission electron microscopy (TEM) were obtained by bead beating a pooled sample of seagrass leaves collected in December 2016 in SM buffer as described above. Homogenates were filtered through a 0.45 μm Sterivex filter (Millipore), and approximately 8 mL of filtrate were layered onto 2.5 mL of a 38% sucrose cushion (Hurwitz et al. 2012) prepared in 14 × 95 mm Thinwall, Ultra-Clear™ ultracentrifuge tubes (Beckman Coulter) and brought to a total of 14 mL by adding 0.02 μm filtered SM buffer. Samples were ultracentrifuged at 175,000 × g for 3 h and 15 min at 18°C. The supernatant was gently poured off and the viral pellet was air dried in a hood for 15 min, after which it was resuspended in 50 μL of 0.02 μm filtered SM buffer.

Five microliters of these purified VLPs were deposited onto a carbon coated formvar grid (Electron Microscopy Sciences) and allowed to absorb for 30 min. Grids were stained using 0.02 μm filtered 2% aqueous uranyl acetate negative stain by placing three successive drops on the grid held with tweezers at a 45° angle and letting the stain fall into a waste container below. Grids were then immersed sample-side up at the bottom of a stain drop for 45 s before being wicked dry from the edge with a wedge of Whatman filter paper. After drying for 1 h in the hood, the grids were examined in a Hitachi 7100 TEM (Hitachi) at 100 kV. Images were recorded using a Gatan Orius high-resolution digital camera.

Results and discussion
Viral genome and phylogeny
We discovered TVX, a novel positive-sense ssRNA virus, in apparently healthy *T. testudinum* seagrasses collected from
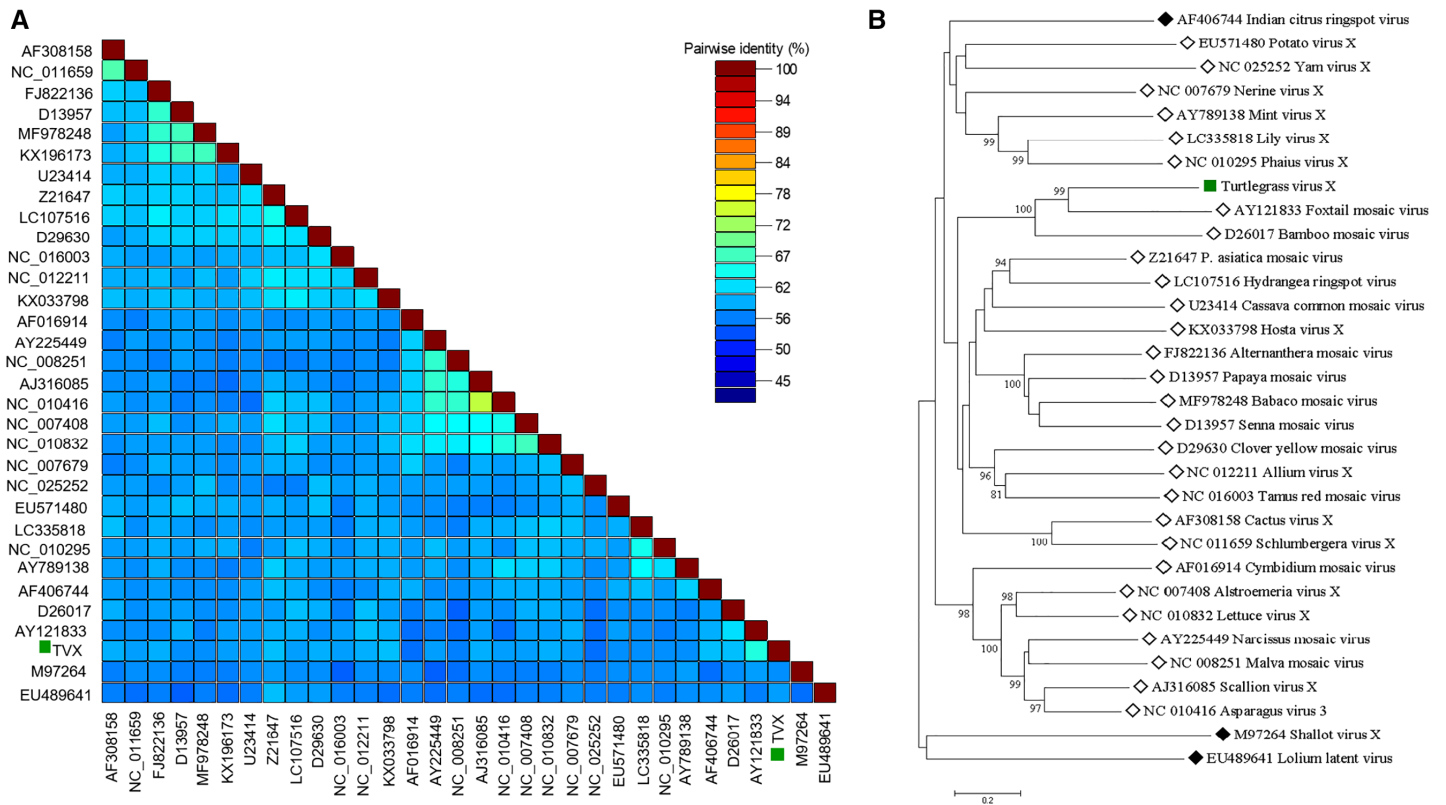
Fig. 3. Analysis of relationships among members of the genus *Potexvirus* (empty diamonds) and other members of the *Alphaflexiviridae* (filled diamonds) based on genome-wide pairwise identities **(A)** and phylogenetic analysis of RdRP amino acid sequences **(B)**. TVX is represented by a green rectangle in both panels. Genome-wide pairwise identities are shown in a color-coded matrix, and accession numbers match viral species names provided on the neighbor joining phylogenetic tree. For each isolate, the GenBank accession number and species name are indicated. Bootstrap values below 75 are not shown.

Tampa Bay, Florida, in December 2016 through SISPA. Preliminary sequence analysis of SISPA products revealed that TVX was most closely related to the potexvirus FoMV. Besides TVX, no other viral sequences were found among the sequenced SISPA products. We completed the TVX genome sequence through primer walking and RACE (Supporting Information Table S2).

The linear TVX genome, 6252 nt in length, is predicted to encode five different proteins: the RdRP, three movement proteins known as the triple gene block, and the capsid (Fig. 2). In addition, the 5′ noncoding region of the TVX genome begins with the sequence "GAAAA," which has been proposed to be an essential motif for RNA replication (Chen et al. 2005). This genome organization coupled with phylogenetic analysis (Fig. 3) indicates that TVX belongs to genus *Potexvirus* (family *Alphaflexiviridae*, Order *Tymovirales*). The genus *Potexvirus* contains more than 30 species of viruses that infect terrestrial plants. All potexviruses contain positive-sense ssRNA genomes encapsidated within flexuous and filamentous virions (Adams et al. 2012). Current species demarcation criteria for the genus *Potexvirus* state that distinct species share < 72% nucleotide (or < 80% amino acid) identity over the RdRP- or capsid-

encoding regions (Adams et al. 2012). With its RdRP and capsid proteins each sharing only 65% amino acid identity with the most closely related potexvirus (FoMV), TVX represents a new species within the genus.

Genome-pairwise identities and phylogenetic analysis indicate that TVX is most closely related to FoMV and bamboo mosaic virus, sharing 66% and 61% identity with these terrestrial grass-infecting viruses, respectively. Analysis of genome-wide pairwise identities among current and proposed members of the genus *Potexvirus* supports TVX as a new species since distinct potexvirus species share < 70% identity (Fig. 3).

Prevalence and viral load

We developed a qPCR assay to determine the prevalence and viral load of TVX in seagrass leaves collected in February 2017 ($n = 40$) and in August 2017 ($n = 40$) from four different sites in Tampa Bay (Fig. 1). The detection limit of the assay, as assessed by running triplicates of the standard curve and obtaining between 97% and 100% efficiency, was equal to a Cq-value of 32 (4.55×10^2 TVX RdRP gene copies mg^{-1} seagrass). Hence, we considered samples with Cq-values greater than or equal to 32 as negative.
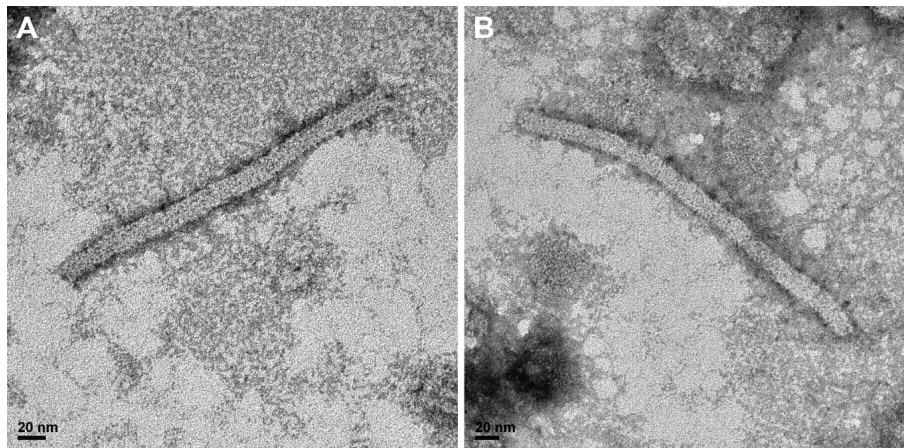
Fig. 4. TEM images of viral preparations from *T. testudinum* samples. **(A and B)** Two examples of filamentous VLPs found in samples collected in December 2016 are shown.

We only detected TVX in seagrass leaves collected in sites N01 and N02, with N01 having the highest viral prevalence. We detected TVX in 80% and 60% of seagrass leaves tested from site N01 in February and August, respectively, whereas 10% and 20% of the leaves from the N02 site were positive in these months. Viral load, which we calculated as the number of TVX RdRP gene copies mg^{-1} seagrass, was higher in summer (August) than in winter (February) for both sampling locations. In February, we detected an average of 2.27×10^8 ($\pm\ 1.86 \times 10^8$ SD) copies mg^{-1} and 2.33×10^6 copies mg^{-1} in leaves from the N01 and N02 sites, respectively. In August, TVX viral load was slightly higher with 1.53×10^9 ($\pm\ 3.07 \times 10^9$ SD) copies mg^{-1} and 1.00×10^8 copies mg^{-1} ($\pm\ 4.45 \times 10^7$ SD) detected in leaves from the N01 and N02 sites, respectively. However, the difference in viral load between February and August was not statistically significant (Student *t*-test, $p = 0.36$). TVX was not detected in sites N03 or N04 at either time point nor was this virus detected in any of the samples collected from Florida Bay in May 2017. These results show that TVX is present in high concentrations but only in a limited area (N01–N02). Future sampling campaigns in this area will focus on investigating virus ecology, including seasonal variation in viral load and prevalence, potential virus transmission routes, and effects on seagrass health.

Transmission electron microscopy

Using TEM, we observed various filamentous VLPs resembling typical potexvirus morphologies in viral preparations obtained from seagrass leaves taken in December 2016 (Fig. 4). These VLPs consistently averaged 20 nm in diameter but varied in length, averaging 224 nm ($\pm$ 92.5 SD, $n = 16$). Although the observed diameter is similar to that of flexuous filaments typical of members of the genus *Potexvirus* (13 nm), the average length is considerably smaller as members of this group range between 470 and 580 nm (Adams et al. 2012). The shorter size when compared to known potexvirus virions

combined with the observed variable lengths suggests that the observed VLPs from seagrass samples were damaged during sample preparation. Therefore, we cannot report an estimated length for the observed filamentous virions, which potentially represent TVX. This filamentous morphotype was the only virus-like morphology observed by TEM.

Ecological importance

Despite the omnipresence and high abundance of viruses in the oceans (reviewed in Breitbart 2012), there is a paucity of information regarding viruses infecting seagrasses, an important group of marine autotrophs. Prior to our study, only a single dsRNA virus had been described from *Z. marina* (eelgrass; Fukuhara et al. 2006); thus, the description of TVX from *T. testudinum* represents a substantial advance in knowledge. Furthermore, TVX represents the first report of a capsid-encoding virus infecting seagrasses, as the dsRNA virus described in eelgrass belongs to the genus *Endornavirus*, a group that does not produce virions (Fukuhara and Gibbs 2012). Notably, endornaviruses would not have been recovered with the methods utilized in our study, which employed purification of VLPs prior to sequencing. The detection of TVX supports that the scarcity of viral discoveries in seagrasses likely reflects the absence of scientific attention, or constraints in the detection of viruses, rather than a high resistance of seagrasses against infection (Duarte et al. 2004).

We detected TVX in apparently healthy *T. testudinum* meadows. This is in line with known members of the genus *Potexvirus*, which for the most part are only mildly pathogenic and cause little damage to host plants (Adams et al. 2012). It is important to note that viruses often coexist with plants without causing disease, displaying a form of mutualism (Roossinck 2011). Although viruses are commonly perceived as pathogens, many viruses are in fact beneficial to their hosts in various ways, for instance, by providing thermal tolerance

or cross-protection against other microorganisms. Most information on terrestrial plant viruses has been acquired from symptomatic cultivated hosts rather than asymptomatic native or wild species, despite the importance of native species as reservoirs of viral diversity (Wren et al. 2006). Recently, enhanced systematic surveys of apparently healthy plants has led to the discovery of numerous novel viruses for which the ecological impact is not yet known (Roossinck 2012). In seagrasses, potential effects caused by viral infection could be negative (e.g., disease, growth reduction), positive (e.g., conferring tolerance to environmental conditions, inducing protection against grazers), or neutral, with the possibility of variable future effects upon changing environmental conditions. However, further sampling and research is required to resolve the exact nature of the virus–host interaction between TVX and *T. testudinum.*

Overall, little is known regarding the influence of associated microbial communities on the health of marine plants or which factors affect the composition of the marine plant microbiome (Ettinger et al. 2017). We speculate that the "seagrass virome," an overlooked part of the seagrass microbiome, is important for seagrass health and function. In this respect, the discovery of TVX in *T. testudinum* represents a first and important step in the study of seagrass–virus interactions. Future research should focus on unraveling the ecology (transmission pathways) and (co)evolution of TVX and its host in order to better predict the potential effects of viruses on seagrasses.

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