JOURNAL OF GENERAL VIROLOGY

SHORT COMMUNICATION
Lim *et al.*, *Journal of General Virology* 2024;105:002004
DOI 10.1099/jgv.0.002004

MICROBIOLOGY SOCIETY
OPEN ACCESS

Limited potexvirus diversity in eastern Gulf of Mexico seagrass meadows

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Abstract

Turtlegrass virus X, which infects the seagrass *Thalassia testudinum*, is the only potexvirus known to infect marine flowering plants. We investigated potexvirus distribution in seagrasses using a degenerate reverse transcription polymerase chain reaction (RT-PCR) assay originally designed to capture potexvirus diversity in terrestrial plants. The assay, which implements Potex-5 and Potex-2RC primers, successfully amplified a 584 nt RNA-dependent RNA polymerase (RdRp) fragment from TVX-infected seagrasses. Following validation, we screened 74 opportunistically collected, apparently healthy seagrass samples for potexviruses using this RT-PCR assay. The survey examined the host species *T. testudinum*, *Halodule wrightii*, *Halophila stipulacea*, *Syringodium filiforme*, *Ruppia maritima*, and *Zostera marina*. Potexvirus PCR products were successfully generated only from *T. testudinum* samples and phylogenetic analysis of sequenced PCR products revealed five distinct TVX sequence variants. Although the RT-PCR assay revealed limited potexvirus diversity in seagrasses, the expanded geographic distribution of TVX shown here emphasizes the importance of future studies to investigate *T. testudinum* populations across its native range and understand how the observed fine-scale genetic diversity affects host-virus interactions.

Impact statement

Potexviruses are widespread in terrestrial plants; however, the recent discovery of TVX in the seagrass *Thalassia testudinum* extends their host range to marine flowering plants. Here we use existing Potex-5 and Potex-2RC degenerate primers to explore potexvirus infections in several seagrass species. TVX sequence variants were detected in *T. testudinum* collected from the eastern Gulf of Mexico, uncovering previously unknown genetic diversity of this poorly understood virus.

DATA SUMMARY

All sequence data are available in NCBI GenBank under the accession numbers OR827692-OR827705, OR854648, OR863396, OR879052-OR879056, and PP430548-PP430571. The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

Received 22 March 2024; Accepted 07 June 2024; Published 18 June 2024
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Keywords: *Alphaflexiviridae*; marine; PCR; plant; *Potexvirus*; primer; seagrass.
Abbreviations: AltMV, Alternanthera mosaic virus; BaMV, Bamboo mosaic virus; FoMV, Foxtail mosaic virus; HKY, Hasegawa-Kishino-Yano; HS, High sensitivity; ICTV, International Committee on Taxonomy of Viruses; LolV, Lolium latent virus; MEGA, Molecular Evolutionary Genetics Analysis; nr, non-redundant; nt, nucleotide; PVX, Potato virus X; RdRp, RNA-dependent RNA polymerase; RT-PCR, Reverse transcription polymerase chain reaction; ssRNA, Single-stranded RNA; TVX, Turtlegrass virus X; Zymo Research, ZR.
All sequence data have been deposited into NCBI GenBank under the accession numbers OR827689-OR827705, OR854648, OR863396, OR879052-OR879056, and PP430548-PP430571.
A supplementary table is available with the online version of this article.

INTRODUCTION

Potexviruses are positive-sense single-stranded RNA (ssRNA) viruses from the family *Alphaflexiviridae* that infect many agronomically important flowering plants (angiosperms), including potato, tobacco, and tomato [1]. The type member of this genus is *Potato virus X* (PVX), from which the genus name *Potexvirus* is derived [2]. Potexviruses have a broad geographic range that reflects the distribution of their hosts and the prevalence of global commerce [2, 3]. Although each potexvirus species has a limited natural host range, some species can infect a wide range of host plants in experimental settings [1]. Potexviruses are transmitted mechanically through contaminated agricultural equipment or plant-to-plant contact [1–3]. Transmission via seed and aphid vectors and non-specific transmission through chewing insects has been reported but is less common [1–3]. Most potexviruses cause persistent, asymptomatic infections or mild mosaic disease, while some cause dwarf, necrotic, or ringspot symptoms in their natural hosts [1, 2]. Notably, PVX can cause severe symptoms when its plant host is co-infected with potyviruses [4–6].

In addition to infecting terrestrial plants, potexviruses are also capable of infecting seagrasses, the only marine angiosperms. A novel potexvirus species, *Turtlegrass virus X* (TVX), was discovered in apparently healthy *Thalassia testudinum* seagrass samples collected from Terra Ceia Aquatic Preserve, Tampa Bay, Florida, USA [7]. The identification of TVX indicates that an important aspect of potexvirus diversity has been overlooked by focusing only on terrestrial plants. Degenerate primers used in reverse transcriptase polymerase chain reaction (RT-PCR) assays, such as Potex-5 and Potex-2RC [8], have proven useful in detecting diverse potexviruses, but have not been tested in aquatic habitats. This study applied the Potex-5 and Potex-2RC primer pair in a RT-PCR assay to explore potexvirus diversity in samples from six seagrass host species.

METHODS

Primer validation

Potex-5/Potex-2RC were tested on cDNA samples each synthesized from 1 µg RNA extracted from *Portulaca* sp. infected with *Alternanthera mosaic virus* (AltMV) and *Opuntia* sp. with a mixed infection of potexviruses, as well as cDNA pooled from *T. testudinum* samples known to contain TVX, based on the TVX-specific RT-PCR assay described by [7]. The PCR reaction contained 0.48 µM of each primer, 2 µl cDNA template, 1 µl GC enhancer, and 1X AmpliTaq Gold 360 Master Mix (Applied Biosystems, Waltham, MA, USA) in a 25 µl reaction volume. PCR was performed under the following conditions: initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 51.5°C (as published in van der Vlugt and Berendsen [8]) for 30 s, extension at 72°C for 1 min, followed by elongation at 72°C for 10 min and cooling at 11°C. The PCR product was visualized following gel electrophoresis on a 1% (wt/vol) agarose gel stained with ethidium bromide. All PCR reactions, except for the no template control, yielded visible bands. All PCR products were purified using Zymo Research's (ZR) Zymoclean Gel DNA Recovery Kit (Irvine, CA, USA), quantified using the Qubit DNA high sensitivity (HS) assay (Invitrogen, Waltham, MA, USA), and Sanger sequenced bidirectionally by Eurofins Genomics (Louisville, KY, USA).

RT-PCR

Total RNA extraction was performed on 30–100 mg of leaves from multiple shoots pooled by seagrass species and collection site (Table S1, available in the online version of this article) using ZR's Quick-RNA Plant Miniprep kit. Each pooled seagrass sample was homogenized in a BashingBead Lysis Tube containing 2 mm ceramic beads and 800 µl RNA lysis buffer (provided in the kit) for 5 min at maximum speed using a Fisherbrand Bead Mill 4 Homogenizer (Fisher Scientific, Waltham, MA, USA). Tissue homogenates were centrifuged at maximum speed (21130 ***g***) for 1 min and RNA was extracted from the total volume (~800 µl) of the supernatant. To ensure successful RNA extraction, after each round of extraction, a random subset of RNA samples was quantified using the Qubit RNA HS assay (Invitrogen). From each sample, cDNA was synthesized from 8 µl RNA using the SuperScript IV First-Strand Synthesis System (Invitrogen) and following manufacturer's instructions for random hexamers. PCR amplification was performed on each cDNA sample, followed by gel electrophoresis, PCR product purification, DNA quantification, and Sanger sequencing, using the methods described above.

Amplicon sequence analysis

Amplicon sequences were first compared with sequences from NCBI's nucleotide (nt) and non-redundant (nr) protein collections [9] and the TVX genome [7] using the megablast and/or blastx programmes on NCBI's BLAST server [10]. Sequence reads from each sample were then mapped to the Potex-5/Potex-2RC amplicon region (with forward and reverse primer sequences removed) in the TVX genome [7] using the 'Map Sanger Reads to Reference' function implemented in Unipro UGENE v48.1 [11], with a trimming quality threshold of 20 and mapping minimum similarity of 70%. Electropherograms of mapped reads were manually inspected to check for mixed infection of potexviruses, to remove primer sequences and low-quality bases, and to resolve ambiguous bases.

Sequences from 42 Potex-5/Potex-2RC PCR products amplified from seagrass and terrestrial plant samples described above were aligned with corresponding amplicon regions (excluding primer sequences) extracted from the reference genomes of TVX

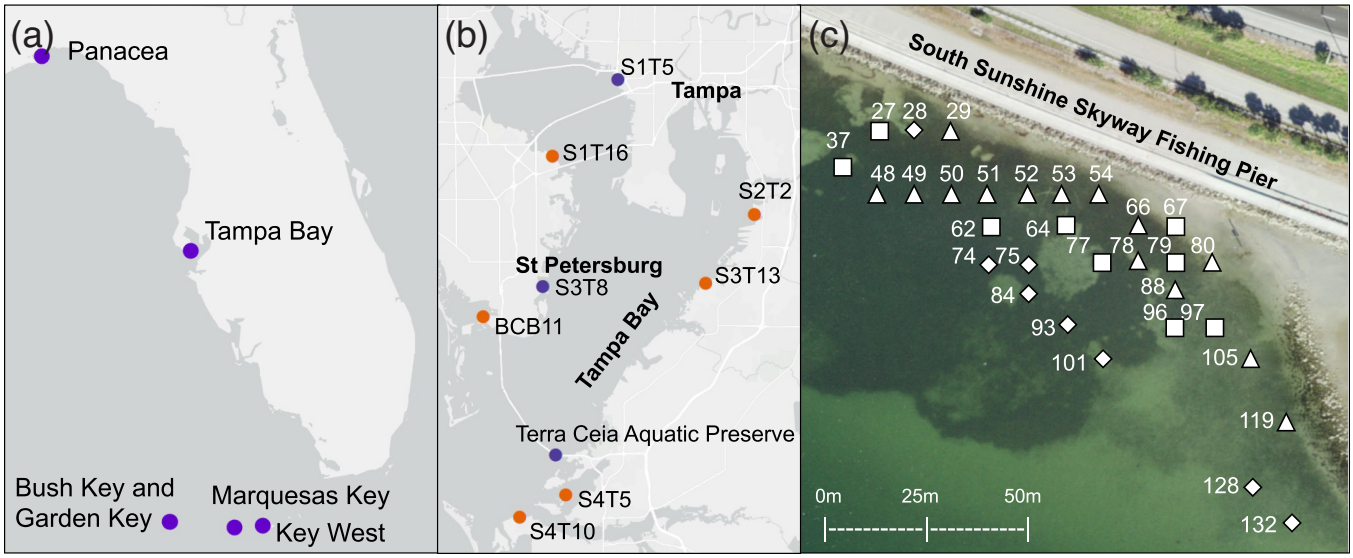

Fig. 1. Map showing locations of seagrass sites in (a) Florida, (b) Tampa Bay, and (c) Terra Ceia Aquatic Preserve where samples were collected for RT-PCR. Purple markers in (a) and (b) denote sites where TVX products were amplified from *T. testudinum* with the Potex-5/Potex-2RC primers, whereas samples from sites with orange markers tested negative. Marker shapes in (c) represent potexvirus subclades found in Terra Ceia Aquatic Preserve sampling sites and correspond to Fig. 2 (negative samples not shown). Metadata of seagrass samples are described in Table S1.

(NC_040644) [7], *Bamboo mosaic virus* (NC_001642) [12], *Foxtail mosaic virus* (NC_001483) [13], AltMV (OR607766), PVX (NC_011620) [14], and *Lolium latent virus* (NC_010434) [15] retrieved from the NCBI Virus database [16]. Multiple sequence alignment was performed using the L-INS-I and --adjustdirectionaccurately options in MAFFT v7.5.08 [17], then trimmed to 514 nt to remove leading and trailing gaps. Pairwise sequence identities were calculated from the trimmed alignment using Clustal Omega v1.2.3 [18] with the --distmat-out, --percent-id, and --full options. For phylogenetic analysis, Molecular Evolutionary Genetics Analysis (MEGA) 11 [19] identified the best model for the trimmed alignment to be the Hasegawa-Kishino-Yano model [20] with a discrete Gamma distribution (HKY+G). Using this substitution model, a maximum likelihood tree with 1000 bootstrap replicates was constructed from the alignment using MEGA11 [19]. The final tree was visualized together with the heatmap of pairwise sequence identity using the ggtree R package v3.6.2 [21].

RESULTS

The Potex-5/Potex-2RC primer pair successfully amplified TVX from *T. testudinum* samples collected from Terra Ceia Aquatic Preserve, AltMV infecting *Portulaca* sp., and an uncharacterized potexvirus infecting *Opuntia* sp. [8]. A RT-PCR assay using Potex-5/Potex-2RC was subsequently performed on seagrass leaves opportunistically collected from separate sampling efforts, including 1) *T. testudinum*, *H. wrightii*, *S. filiforme*, and *R. maritima* from Tampa Bay on 26 Jan or 4 Feb 2022; 2) *T. testudinum* from the Dry Tortugas National Park, Florida, USA collected between 16–20 May 2022; 3) *T. testudinum* from a systematic seagrass survey at Terra Ceia Aquatic Preserve on 1 August 2022; and 4) *T. testudinum*, *H. wrightii*, and *S. filiforme* samples collected from Tampa Bay seagrass site S3T8 (Lassing Park) on 3 October 2023 (Fig. 1 and Table S1). Seagrass samples (Table S1) were also received from other research groups, including *T. testudinum* from Panacea (Florida, USA), *Zostera marina* from York River (Virginia, USA), West Falmouth Harbour (Massachusetts, USA), Sitka (Alaska, USA), New Zealand, and Kalmar (Sweden), and *Halophila stipulacea* from Jobos Bay National Estuarine Research Reserve (Puerto Rico, USA). PCR products were successfully amplified and sequenced from 40 *T. testudinum* samples from Florida sites, including Terra Ceia Aquatic Preserve, Tampa Bay seagrass sites S1T5 and S3T8 (Lassing Park), Panacea located in the Florida Panhandle, and Florida Keys sites including Bush Key, Garden Key, Marquesas Key, and Key West (Fig. 1). Based on our analysis of Sanger sequencing traces, no evidence of mixed potexvirus infection was found in these *T. testudinum* samples. Aside from *T. testudinum*, no other seagrass species produced PCR products.

Phylogenetic analysis placed all Potex-5/Potex-2RC amplicons from seagrasses in the same clade as the RdRp sequence fragment from the TVX genome [7] with 100% bootstrap confidence (Fig. 2a). Sequences within this clade shared 74.4–98.6% nucleotide sequence identities to each other (Fig. 2b). TVX sequences from Terra Ceia Aquatic Preserve formed three subclades. Average pairwise nucleotide sequence identities were 95.8±1.4% within each subclade and 79.3±2.9% between subclades (Fig. 2). Two Terra Ceia Aquatic Preserve subclades, including the subclade containing the amplicon region from the TVX genome, were sister

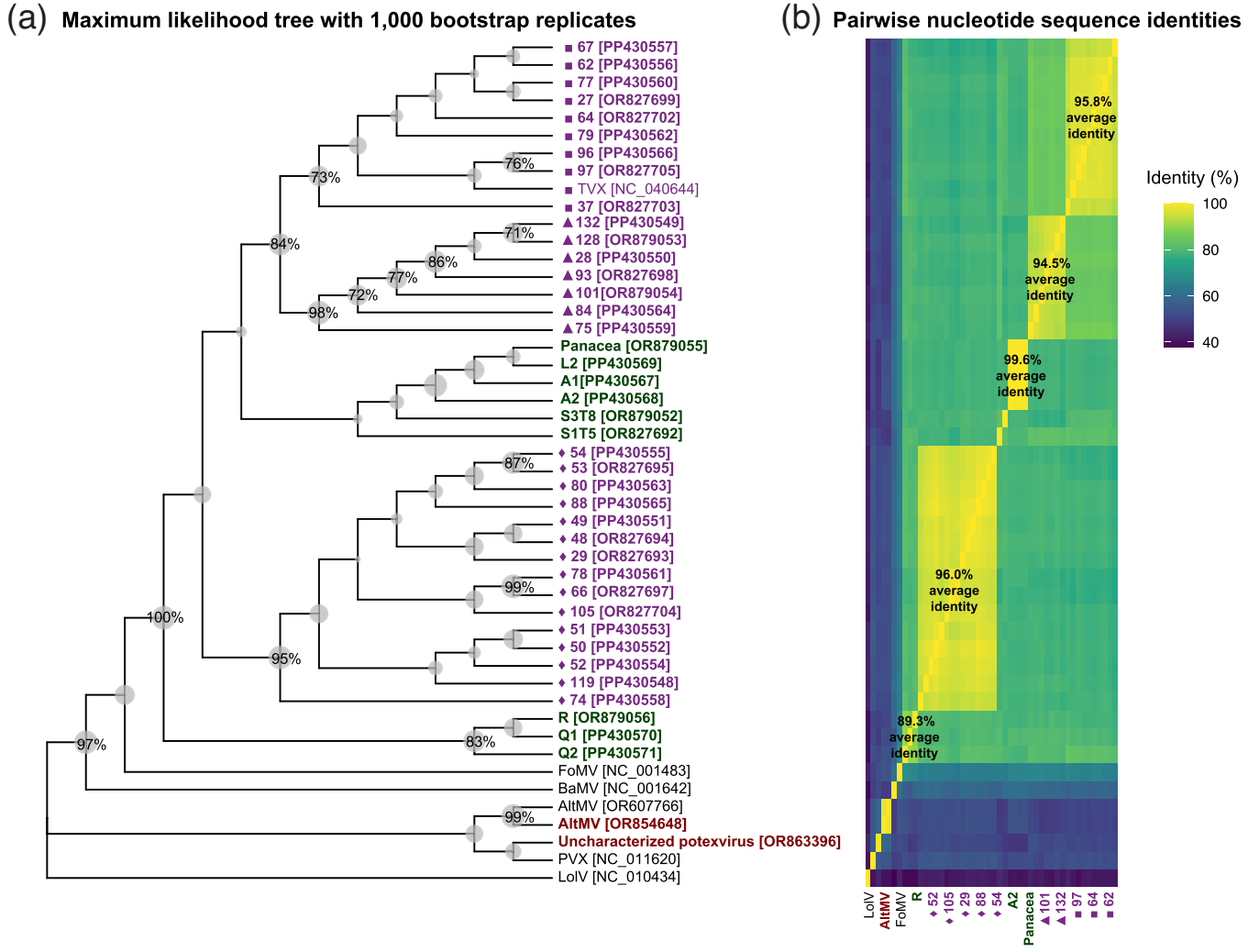

Fig. 2. (a) Bootstrap maximum likelihood tree and (b) heatmap of pairwise nucleotide sequence identities of TVX nucleotide sequences amplified from seagrasses collected from Terra Ceia Aquatic Preserve (purple text) and other sites in the eastern Gulf of Mexico (green text), in relation to potexvirus sequences amplified from terrestrial plants (brown text) and potexvirus RdRp sequences from reference genomes (black, not-bold text). The NCBI accession number for each sequence in (a) is indicated in square brackets. The tree in (a) was constructed by MEGA11 from a 514 nt conserved region in the multiple sequence alignment. Sizes of tree nodes are proportionate to bootstrap values indicating the percentage of trees, based on 1000 replicates, in which sequences within a node were clustered together. Only bootstrap values >70% are labelled on the tree nodes. *Lolium latent virus* was used as an outgroup for the tree. Marker shapes in (a) and (b) preceding sequences in the Terra Ceia Aquatic Preserve subclades correspond to marker shapes in Fig. 1c. Abbreviations: TVX, *Turtlegrass virus X*; AltMV, *Alternanthera mosaic virus*; PVX, *Potato virus X*; LolV, *Lolium latent virus*; FoMV, *Foxtail mosaic virus*; BaMV, *Bamboo mosaic virus*.

groups to each other, sharing 84.6±1% average pairwise nucleotide sequence identities (Fig. 2). Other TVX sequences from Key West (A1 and A2), Marquesas Key (L2), Upper Tampa Bay (S1T5 and S3T8), and Panacea formed a subclade with 99.6±0.2% average pairwise nucleotide sequence identities, while sequences from Garden Key (Q1 and Q2) and Bush Key (R) formed another subclade with 89.3±0.9% average pairwise nucleotide sequence identities (Fig. 2). Potexviruses that infect terrestrial grasses (*Foxtail mosaic virus* and *Bamboo mosaic virus*) formed sister clades to the TVX-containing clade (Fig. 2a).

DISCUSSION

In this study, we validated the ability of the potexvirus degenerate primers, Potex-5/Potex-2RC, published by van der Vlugt and Berendsen [8], to amplify TVX-infected seagrass samples and potexvirus-infected terrestrial plant samples. Potex-5/Potex-2RC were used in a RT-PCR survey to test six seagrass species for potexviruses. Among the seagrass species examined, potexvirus infection was only detected in *T. testudinum*, the species from which TVX was first identified. The criteria published by the International Committee on Taxonomy of Viruses (ICTV) demarcates potexviruses into species based on host range, absence

of cross-protection in infected plants, serology, and sequence identity thresholds of 72% for nucleotide sequences and 80% for amino acid sequences (https://ictv.global/report/chapter/alphaflexiviridae/alphaflexiviridae/potexvirus). Potexviruses amplified in this study are specific to *T. testudinum* and shared >72% sequence identity to each other and to the RdRp sequence fragment from the TVX genome [7]. Therefore, we considered identified potexviruses the same species as TVX. Note that this study only analysed a short and conserved fragment of the RdRp sequence and these pairwise identities may change when considering complete genomes. Nevertheless, our results suggest limited potexvirus diversity in marine angiosperms. RT-PCR analysis revealed *T. testudinum* as a natural host for TVX and confirmed TVX infection in eastern Gulf of Mexico seagrass meadows, including those in northwest (Panacea), west-central (Tampa Bay), and southwest (Florida Keys) Florida. Previous phylogenetic analyses of the PVX coat protein and genomic sequences identified phylogroups that reflect intraspecies diversity [22, 23]. Here, three distinct TVX phylogroups, sharing >95% intra-group and <80% inter-group amplicon sequence identity, were observed within a single seagrass meadow at Terra Ceia Aquatic Preserve, along with two additional phylogroups in Upper Tampa Bay, the Florida Keys, and Panacea.

Among all sites sampled in this study, the most extensive TVX survey was conducted within the Terra Ceia Aquatic Preserve. Systematic RT-PCR surveys at other sites will be useful in elucidating potexvirus diversity in seagrasses. The sampling scheme in this study was limited to *T. testudinum* populations along the eastern Gulf of Mexico. However, *T. testudinum* has a widespread geographic range across the western Atlantic Ocean with high genetic diversity that is generally partitioned into two clusters corresponding to the Gulf of Mexico or the Caribbean phylogeographic regions [24]. Although seagrasses can reproduce asexually, some *T. testudinum* populations, such as those in Tampa Bay [25–27] and Florida Bay [28, 29], undergo sexual reproduction and exhibit high genotypic diversity. Currently, the relationship between *T. testudinum* genotype and TVX diversity is unknown and should be further investigated. Future potexvirus surveys covering the total distribution range of *T. testudinum*, the related species *T. hemprichii*, and other seagrass species also have the potential to uncover relationships between potexvirus diversity, host diversity, and their phylogeographic distributions.

Funding information
This project was supported by award OCE-2219547 from the National Science Foundation to M.B., K.R.C., and B.T.F. and a Proposal Enhancement Grant from the University of South Florida Internal Awards Programme to M.B. This research was made possible by support from the Florida Institute of Oceanography for collection of the Dry Tortugas samples.

Acknowledgements
Fieldwork was conducted under permit no. FKNMS-2022–026 issued by the Florida Keys National Marine Sanctuary, permit no. DRTO-2022-SCI-0004 issued through the U.S. Department of the Interior National Park Service and permit no. SAL-19–1172 A-SR issued through the Florida Fish and Wildlife Conservation Commission. We acknowledge the contributions of Luis Alvarado-Marchena, Victoria Congdon, Keith Keel, Isabella Ritchie, Taylor Little, Jessye Kirkham, and Mike Wheeler in field work and sample collection at these sites. Isabella Ritchie provided cDNAs from *T. testudinum* samples from Terra Ceia Aquatic Preserve. Salvador P Lopez, Jr and Scott Adkins from the Agricultural Research Service, United States Horticultural Research Laboratory, U.S. Department of Agriculture (USDA), Fort Pierce, FL, USA provided cDNAs from potexvirus-infected plant samples. We thank Maya Groner, Ian Hewson, Karin Holmfeldt, Natalia Lopez Figueroa, Carolyn Malmstrom, Jordan Rede, Cypress Rudloe, and Jack Rudloe for providing seagrass samples from other sites.

Author contributions
S.J.L.: Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Visualization, Writing - original draft. K.R.: Conceptualization, Formal analysis, Funding acquisition, Writing – review & editing. M.E.K.: Investigation, Writing – review & editing. A.J.G.: Investigation, Writing - review & editing. B.T.F.: Funding acquisition, Investigation, Writing - review & editing. M.B.: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing - review & editing.

Conflicts of interest
The author(s) declare that there are no conflicts of interest.

References

1. Ryu KH, Hong JS, Mahy BWJ, Van Regenmortel MHV. Potexvirus. In: *Encyclopedia of Virology*, 3rd edn. Oxford: Academic Press, 2008. pp. 310–313.
2. Verchot J. *Potato virus* X: a global potato-infecting virus and type member of the *Potexvirus* genus. *Mol Plant Pathol* 2022;23:315–320.
3. AbouHaidar MG, Gellatly D. Potexviruses. In: Granoff A and Webster RG (eds). *Encyclopedia of Virology*, 2nd edn. Oxford: Elsevier; 1999. pp. 1364–1368.
4. Rochow WF, Ross AF. Virus multiplication in plants doubly infected by potato viruses X and Y. *Virology* 1955;1:10–27.
5. Pacheco R, García-Marcos A, Barajas D, Martiáñez J, Tenllado F. PVX-potyvirus synergistic infections differentially alter microRNA accumulation in *Nicotiana benthamiana*. *Virus Res* 2012;165:231–235.
6. Pollari M, Sipari N, Poque S, Himanen K, Mäkinen K. Effects of poty-potexvirus synergism on growth, photosynthesis and metabolite status of *Nicotiana benthamiana*. *Viruses* 2022;15:121.
7. Van Bogaert N, Rosario K, Furman BT, Hall MO, Greco AM, *et al*. Discovery of a novel potexvirus in the seagrass *Thalassia testudinum* from Tampa Bay, Florida. *Limnol Oceanogr Lett* 2019;4:1–8.
8. van der Vlugt RAA, Berendsen M. Development of a general potexvirus detection method. *Eur J Plant Pathol* 2002;108:367–371.
9. Sayers EW, Beck J, Bolton EE, Bourexis D, Brister JR, *et al*. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res* 2021;49:D10–D17.
10. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, *et al*. NCBI BLAST: a better web interface. *Nucleic Acids Res* 2008;36:W5–W9.
11. Okonechnikov K, Golosova O, Fursov M. Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics* 2012;28:1166–1167.

12. Lin NS, Lin BY, Lo NW, Hu CC, Chow TY, *et al*. Nucleotide sequence of the genomic RNA of bamboo mosaic potexvirus. *J Gen Virol* 1994;75:2513–2518.
13. Bancroft JB, Rouleau M, Johnston R, Prins L, Mackie GA. The entire nucleotide sequence of foxtail mosaic virus RNA. *J Gen Virol* 1991;72:2173–2181.
14. Huisman MJ, Linthorst HJM, Bol JF, Cornelissen BJC. The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. *J Gen Virol* 1988;69:1789–1798.
15. Vaira AM, Maroon-Lango CJ, Hammond J. Molecular characterization of *Lolium latent virus*, proposed type member of a new genus in the family *Flexiviridae*. *Arch Virol* 2008;153:1263–1270.
16. Brister JR, Ako-Adjei D, Bao Y, Blinkova O. NCBI viral genomes resource. *Nucleic Acids Res* 2015;43:D571–D577.
17. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013;30:772–780.
18. Sievers F, Higgins DG. Clustal Omega for making accurate alignments of many protein sequences. *Protein Sci* 2018;27:135–145.
19. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Mol Biol Evol* 2021;38:3022–3027.
20. Hasegawa M, Kishino H, Yano T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* 1985;22:160–174.
21. Yu G, Smith DK, Zhu H, Guan Y, Lam T-Y. GGTREE: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *MEE* 2017;8:28–36.
22. Cox BA, Jones RA. Genetic variability in the coat protein gene of *Potato virus X* and the current relationship between phylogenetic placement and resistance groupings. *Arch Virol* 2010;155:1349–1356.
23. Fuentes S, Gibbs AJ, Hajizadeh M, Perez A, Adams IP, *et al*. The phylogeography of *Potato virus X* shows the fingerprints of its human vector. *Viruses* 2021;13:644.
24. van Dijk K-J, Bricker E, van Tussenbroek BI, Waycott M. Range-wide population genetic structure of the Caribbean marine angiosperm *Thalassia testudinum*. *Ecol Evol* 2018;8:9478–9490.
25. Grey WF, Moffler MD. Flowering of the seagrass *Thalassia testudinum* (Hydrocharitaceae) in the Tampa Bay, Florida area. *Aquat Bot* 1978;5:251–259.
26. Durako MJ, Moffler MD. Factors affecting the reproductive ecology of *Thalassia testudinum* (Hydrocharitaceae). *Aquat Bot* 1987;27:79–95.
27. Campanella JJ, Bologna PAX, Carvalho M, Smalley JV, Elakhrass M, *et al*. Clonal diversity and connectedness of turtle grass (*Thalassia testudinum*) populations in a UNESCO Biosphere Reserve. *Aquat Bot* 2015;123:76–82.
28. Bricker E, Waycott M, Calladine A, Zieman JC. High connectivity across environmental gradients and implications for phenotypic plasticity in a marine plant. *Mar Ecol Prog Ser* 2011;423:57–67.
29. Peñalver MM, Durako M, Furman BT, Hall MO. Multiple stressors result in reduced reproductive effort by *Thalassia testudinum* in Florida Bay, USA. *Mar Ecol Prog Ser* 2020;647:65–78.