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Genus *Orthotospovirus* in Costa Rica: A Central American case

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ABSTRACT

Objective/Background. The *Orthotospovirus* genus encompasses a range of economically significant and emerging plant viruses that affect a variety of crops globally. While the prevalence and characteristics of these phytopathogenic viruses are extensively documented in North and South America, their presence in Central America remains comparatively underexplored. This study focuses on Costa Rica, strategically positioned at the nexus of North and South America, to enhance our understanding of orthotospovirus in this region.

Materials and Methods. We analyzed 295 plant samples using enzyme-linked immunosorbent assay (ELISA) to test for the presence of INSV, IYSV, TSWV, and the GRSV/TCSV serogroup. Additionally, a subset (20 samples) underwent further scrutiny through reverse transcription-polymerase chain reaction (RT-PCR) employing both universal and species-specific primers.

Results. Our ELISA results indicated the absence of TSWV and the GRSV/TCSV serogroup. However, the presence of INSV in Costa Rica was substantiated through ELISA, RT-PCR, and partial sequencing, revealing its prevalence in both open-field and greenhouse environments. Despite previous diagnostic reports suggesting the presence of TSWV in Costa Rica, our study did not detect this virus. RT-PCR analysis with degenerate primers also found no evidence of other orthotospovirus species in our samples. The identification of a dominant INSV haplotype, along with three additional variants, suggests the likelihood of at least two independent virus introductions into the region.

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Conclusion. These findings underscore the necessity for more comprehensive surveys and research on orthotospoviruses in Central America to better understand their epidemiology and impact on agriculture.

Keywords: viral symptoms, ELISA, RT-PCR, genetic diversity, INSV, IYSV.

Introduction

The *Orthotospovirus* genus (family Tospoviridae, order Bunyavirales) (Kuhn *et al.,* 2022) represents an emerging group of viruses with a global distribution, affecting various plant species (Pappu *et al.,* 2009). Members of this genus have expanded their distribution or plant host range (Londoño *et al.,* 2012; Naidu *et al.,* 2005; Webster *et al.,* 2015). Several orthotospovirus species are recognized as economically significant plant pathogens, limiting crop production or affecting yield quality. For instance, different reports mention yield losses up to 100 % and economic losses starting from several tens of thousands to millions of US dollars (Daughtrey *et al.,* 1997; Hasegawa and Del Pozo-Valdivia, 2023; Mandal *et al.,* 2012).

The *Orthotospovirus* genus comprises 26 accepted species (Kuhn *et al.,* 2022). *Tomato spotted wilt virus* (TSWV) is the type species and causes a high economic impact on several crops (Pappu *et al.,* 2009). Another significant species within the genus is *Impatiens necrotic spot virus* (INSV) (Daughtrey *et al.,* 1997). Historically, INSV was first recognized as a distinct serotype of TSWV, identified as TSWV-I (De Ávila *et al.,* 1992). *Iris yellow spot virus* (IYSV) is also noteworthy, an *orthotospovirus* of Eurasian origin that has become a limitation in onion bulb and seed production and has rapidly spread throughout the world (Bag *et al.,* 2015; Gent *et al.,* 2006).

Despite considerable research on orthotospovirus biology, management, and interaction with thrips vectors (Montero-Astúa *et al.,* 2016), information on occurrence and sequence data is quite limited from all Central American countries. TSWV and INSV (at that time TSWV-I) were reported by serology from impatiens (*Impatiens* spp.) and lisianthus (*Eustoma* sp.) plants in Costa Rica in the early 1990s (Hsu and Lawson, 1991; Rivera *et al.,* 1990). IYSV was reported in Guatemala (Nischwitz *et al.,* 2007) and Costa Rica (Montero-Astúa *et al.,* 2017). In addition to these scientific publications, information (local publications, dissertations or institutional project reports available online) for TSWV occurrence in Guatemala and Honduras (Cumes Mantanico, 2008; Dardón *et al.,* 1994; Doyle *et al.,* 2002; Espinoza Rivera, 2012; Palmieri, 2012) was found, but sequence data for TSWV isolates from Central America in public databases were not.

In contrast to the Central American situation, several reports have updated the orthotospovirus presence in the Caribbean islands. Outbreaks of *Tomato chlorotic spot virus* (TCSV), a different species of orthotospovirus originally described from Brazil (De Ávila *et al.,* 1993), were reported throughout the Caribbean Basin: Cuba (Martinez-Zubiaur *et al.,* 2016), Dominican Republic (Almeida *et al.,* 2014; Batuman *et al.,* 2014), Haiti (Adegbola *et al.,* 2016), Puerto Rico (De Jensen and Adkins, 2014; Webster *et al.,* 2013); and additionally in Florida (USA) (Londoño *et al.,* 2012; Webster *et al.,* 2015). Moreover, TSWV was confirmed by RT-PCR in the Dominican Republic in 2013 (Martínez *et al.,* 2014).

Orthotospovirus sequence data is essential from all geographic regions and diverse plant hosts to (i) analyze viral diversity globally, (ii) recognize genetically distinct isolates, and (iii) contribute to epidemiological understanding. This information is critical for crop breeding and disease resistance programs. For instance, there are isolates of TSWV capable of overcoming resistance conferred by the Sw-5 and Tsw resistance genes in tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) plants, respectively. Knowledge of the isolates present in an area and sequence comparisons to identify the mutations that allow resistance-breaking are vital inputs for breeding programs (Batuman *et al.,* 2017; Sharman and Persley, 2006).

In this context, the objective of this work was to determine the prevalence of orthotospoviruses in Costa Rica and obtain partial genome sequences to analyze genetic variability. This information updates the occurrence and variability of these plant viruses in Central America. We conducted a study by enzyme-linked immunosorbent assay (ELISA) of virus-like symptomatic plants to increase the chances of detecting orthotospoviruses. A subset of ELISA-positive samples was further examined by reverse transcription-polymerase chain reaction (RT-PCR). No TSWV-positive plants were detected in our samples. Therefore, we also conducted a longitudinal analysis of TSWV and INSV detection in Costa Rica based on diagnostic clinic reports from the Obligate Plant Pathogens and their Vectors Laboratory (LaFOV), Cellular and Molecular Biology Research Center (CIBCM), University of Costa Rica, available since the year 2000. This study provides a foundational update on the presence and variability of orthotospoviruses in Costa Rica, contributing to the broader understanding of these viruses in Central America. The findings suggest a shift in virus prevalence and underscore the importance of continued monitoring and research. Future efforts should focus on detailed surveillance and genetic studies to better manage these plant viruses and address agricultural challenges.

Materials and Methods

Sample Collection Methodology. We collected a total of 295 plant samples representing 20 different plant families (as detailed in Table 1). These samples were sourced from 47 independent locations, including fields, greenhouses, parks, and gardens across five of the seven provinces in Costa Rica. Our primary targets for analysis through Enzyme-Linked Immunosorbent Assay (ELISA) were INSV,

Table 1. Samples collected from five provinces of Costa Rica to analyze the presence of species of Orthotospvirus.

Plant Family	N° samples / province[z]	Total samples	N° Sites	Samples IDs	Tissue
Amaryllidaceae	2 A, 23 C, 17 SJ	42	13	T161-T163, T170-T175, T177-T180, T182, T183, T192, T195-T199, T283-T285, T288-T296, T298-T301, T305, T316, T317, T321	Leaf
Anacardiaceae	1 SJ	1	1	T335	Fruit
Apocynaceae	1 A, 5 C	6	3	T076, T159, T241, T346-T348	Leaf
Asparagaceae	1 A	1	1	T329	Leaf
Asteraceae	6 A	6	2	T051, T053, T325-T328	Leaf
Balsaminaceae	4 A, 3 C	7	2	T028-T031, T061, T261, T262	Leaf
Caricaceae	5 P	5	1	T330-T334	Fruit
Cucurbitaceae	7 A, 3 C, 1 G, 9 P	20	8	T035-T037, T046-, T047, T080, T090, T091, T160, T268, T270, T280, T135-T140, T265, T266	Leaf
Fabaceae	4 A, 2 C, 1 SJ	7	5	T034, T079, T118, T124, T148, T243, T258	Leaf
Gentianaceae	3 C	3	1	T322-T324	Leaf
Iridaceae	4 C	4	1	T184-T187	Leaf
Lamiaceae	3 C	3	1	T156-T158	Leaf
Liliaceae	2 C	2	1	T188-T189	Leaf
Malvaceae	3 A, 1 G, 1 SJ	5	3	T066-T068, T081, T350	Leaf
Orchidaceae	18 A, 27 C	45	2	T012-T027, T055, T056, T214-T240	Leaf
Oxalidaceae	2 A	2	1	T120, T121	Leaf
Plantaginaceae	2 C	2	1	T057, T058	Leaf
Rosaceae	2 A	2	1	T128, T131	Leaf
Solanaceae	71 A, 37 C, 11 P, 12 SJ	131		T001-T011, T032, T033, T038-T045, T048-T050, T054, T059-T060, T062-T065, T069-T075, T077, T078, T082-T089, T092-T104, T106-T112, T114-T116, T119, T122, T134, T165-T169, T176, T181, T194, T202-T205, T209-T213, T245-T251, T253, T263, T264, T267, T271-T279, T302-T304, T306-T313, T320, T336-T338, T341-T345	Leaf and/ or Fruit
Zingiberaceae	1 A	1	1	T339	Leaf

[z] Sampled provinces; A, Alajuela; C, Cartago; G, Guanacaste; P, Puntarenas; SJ, San José.

IYSV, TSWV, and the serogroup consisting of *Groundnut ring spot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV). The collection period spanned from 2013 to 2014, with samples primarily obtained from growers who submitted them to the diagnostic clinic due to the manifestation of viral-like symptoms in their plants. Additionally, proactive visits were made to fields, greenhouses, gardens, or parks with a known history of orthotospovirus occurrence or reports of symptomatic plants. The observed symptoms prompting sample collection included mosaic patterns, chlorotic or necrotic concentric rings or line patterns, leaf deformation, irregular leaf or fruit surface (such as blistering or crinkling), and stunting. On our selection process, particular attention was given to plant species known as hosts for these viruses, such as tomatoes, onions, and peppers. These horticultural crops were more heavily represented in our sample set due to their agricultural significance and the higher probability of detecting the viruses of interest within them. Alongside these, ornamental and various other horticultural plant species were also included in our study to provide a comprehensive overview of the orthotospovirus presence and diversity in Costa Rica.

Sample Preparation and ELISA Screening. For the detection of INSV, IYSV, TSWV, and the GRSV/ TCSV serogroup, we used commercial ELISA reagents from Agdia, Elkhart, IN, adhering to the manufacturer's instructions. The process began with grinding approximately 0.5 g of plant tissue in 1 mL of a general extraction buffer. This buffer's composition included 136 mM NaCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl, 10mM Na2SO3, 2 % (v/v) Tween-20, 2 % (w/v) Polyvinylpyrrolidone MW 40,000, 0.2 % (w/v) chicken egg albumin, and was pH-adjusted to 7.4. Each sample was prepared in three replicates and stored at -35 °C until they were used in the ELISA tests. For the ELISA procedure, samples were tested in duplicate wells. Each plate included two wells of certified commercial positive controls (Agdia) and four wells of negative controls. The absorbance was measured at 405 nm after one hour, and extended to two hours specifically for IYSV, using a Multiskan FC microplate reader (Thermo Scientific, China). A sample was considered positive if its average absorbance value was greater than twice the average of the corresponding negative control wells, as per the criterion established by Sutula *et al.,* 1986. In cases of doubtful results, samples were retested following the same protocol but utilizing an enzyme-conjugate buffer (ECI buffer, Agdia's protocol) both with and without the addition of 5 % skim milk powder. This step was included to mitigate false positives, a method supported by Smith *et al.* (2006).

Longitudinal Analysis of INSV and TSWV Detection in Costa Rica. The diagnostic clinic reports for INSV and TSWV detection in Costa Rica, conducted at the Obligate Plant Pathogens and their Vectors Laboratory (LaFOV), Cellular

and Molecular Biology Research Center (CIBCM), University of Costa Rica, available since 2000, represent our primary data source. This 23.5-year span of records (2000 to July 2023) offers the most comprehensive insight we have into the detection frequency of INSV and TSWV in the country. The samples submitted to the diagnostic clinic fall into two categories: (i) symptomless plant material intended for export, which requires phytosanitary certification and is likely virus-free, and (ii) symptomatic plant material, typically submitted by growers and extension agents, which is likely virus-infected, though not exclusively with orthotospoviruses. These records, encompassing both healthy and diseased-looking samples from various sizes of farms and greenhouses, provide a valuable estimate of the relative frequency of virus occurrence in Costa Rica over the years.

Refinement of impatiens necrotic spot virus detection via RT-PCR: To enhance the accuracy of INSV identification, we conducted a targeted analysis using RT-PCR on 20 samples that tested positive for INSV via ELISA. This in-depth analysis involved the use of both broad-spectrum orthotospovirus primers and specific primers designed for INSV, as outlined in Table 2 and Figure 1. The RNA was extracted from each sample using the RNeasy Plant Minikit (Qiagen, Germany). Subsequently, we quantified the total RNA using a NanoDrop 2000C UV-Vis spectrophotometer (ThermoScientific, USA) to ensure adequate concentration for the subsequent steps. Then, we prepared the first strand cDNA from 5 µL of the extracted RNA for each sample, achieving a final reaction volume of 20 µL. This synthesis was performed using either the RevertAid H Minus First Strand cDNA Synthesis Kit or Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania), using random hexamers. The synthesized cDNA was then stored at -35 °C for preservation. To ascertain the integrity of the cDNA and to check for potential amplification inhibitors or RNA degradation, we utilized primers targeting a 193 bp fragment of the plant 18S ribosomal RNA subunit, serving as a reference gene for plant gene expression studies (referenced from Zhu *et al.,* 2012). The PCR mixtures, totaling 25 µL each, included 2 µL of the synthesized cDNA, 12.5 µL of DreamTaq PCR Master Mix 2X (Thermo Scientific), and 200 nM of each primer. The precise thermocycling conditions for each primer pair were as per specifications listed in Table 2, using advanced thermocyclers like PTC-200 (MJ Research, Canada), MJ Mini (BioRad, Singapore), and Corbett Research CG1-96 (Corbett Life Science, Australia). After PCR, the products were visualized on a UV light transilluminator (55W, BXT-26M, Uvitec, France). This was done following electrophoresis in a 1% agarose gel with TAE 1X buffer, with the gel stained using GelRed 10000X (Biotium Inc., USA) incorporated into the loading buffer (6X Thermo Scientific) for optimal visualization. To capture a comprehensive genetic snapshot, various primer pairs were employed to sequence different segments of the orthotospovirus genome: small (S), medium (M), and large (L). This approach,

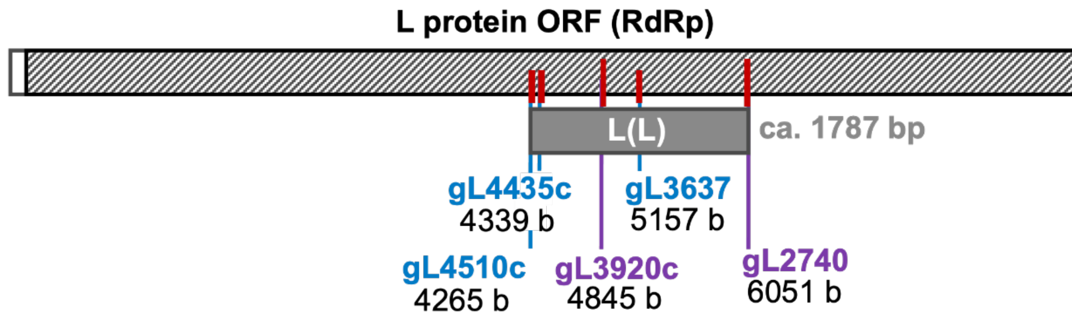
Table 2. Primer pairs and thermocycle profiles used for the detection of orthotospoviruses.

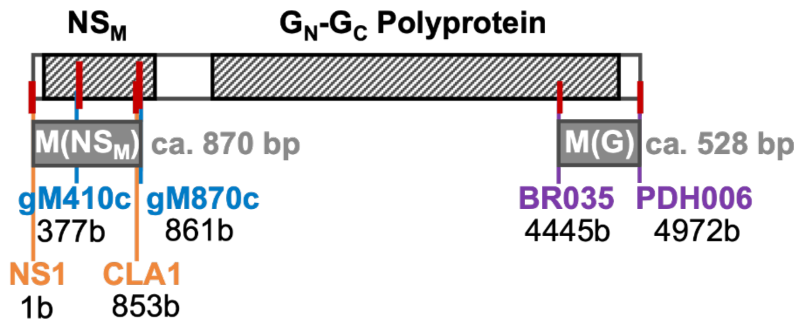
Primer pair	Primer sequence 5' to 3'	Amplicon size (bp)	Thermocycle profile	Reference
Orthotospovirus detection (several species)				
BR60 BR65	CCCGGATCCTGCAGAGCAAATGTGTCA ATCAAGCCTTCTGAAAGTCAT	453	95 °C x 1 min; 30 x (94 °C x 30 s, 48 °C x 1 min, 72 °C); 72 °C x 7 min	Eiras *et al.*, 2001
BR035 PDH006	GAATATATGACACCATTG CCCAGAGCAATCAGTGCA	514	95 °C x 1 min; 30 x (94 °C x 30 s, 48 °C x 1 min, 72 °C); 72 °C x 7 min	Eiras *et al*., 2001
NS1 CLA1	CCCTGCAGGATCCAGAGCAATCAGTGCA GCAGGCTTCAATGAATGC	865	95 °C x 1 min; 30 x (94 °C x 30 s, 48 °C x 1 min, 72 °C x 1 min); 72 °C x 7 min	Eiras *et al*., 2001
gM410 gM870c	AACTGGAAAAATGATTYNYTTGTTGG ATTAGYTTGCAKGCTTCAATNAARGC	500	95 °C x 1 min; 35 x (94 °C x 30 s, 52 °C x 30 s, 72 °C x 30 s); 72 °C x 10 min	Chen *et al*., 2012
gL2740 gL3920c	ATGGGDATNTTTGATTTCATGRTATGC TCATGCTCATSAGRTAAATYTCTCT	1200	95 °C x 2 min; 30 x (94 °C x 30 s, 50 °C x 30 s, 72 °C x 2 min); 72 °C x 5 min	Chen *et al*., 2012
gL3637 gL4435c	CCTTTAACAGTDGAAACAT CATDGCRCAAGARTGRTARACAGA	810	95 °C x 2 min; 30 x (94 °C x 30 s, 50 °C x 30 s, 72 °C x 2 min); 72 °C x 5 min	Chu *et al*., 2001
gL3637 gL4510c	CCTTTAACAGTDGAAACAT TCATCRGARTGBACMATCCATCT	890	95 °C x 2 min; 30 x (94 °C x 30 s, 50 °C x 30 s, 72 °C x 2 min); 72 °C x 5 min	Chu *et al*., 2001
INSV specific detection				
INSV-589 TOS-R15	CCCAAGACACAGGATTTCA GGGAGAGCAATYGWGKYR	589	95 °C x 2 min; 35 x (94 °C x 30 s, 54 °C x 30 s, 72 °C x 1 min); 72 °C x 5 min	Uga & Tsuda, 2005
Plants' 18S ribosomal RNA subunit				
18S-F 18S-R	TCTGCCCGTTGCTCTGATGAT CCTTGGATGTGGTAGCCGTTT	193	95 °C x 1 min; 40 x (94 °C x 30 s, 55 °C x 30 s, 72 °C x 35 s); 72 °C x 5 min	Zhu *et al*., 2012

sometimes involving overlapping amplicons (as depicted in Figure 1), enabled us to acquire partial sequences of four distinct orthotospovirus ORFs (illustrated in Figure 1): the S(N) segment, M(NSM) segment, M(G) segment, and the L(L) segment. These sequences encompass critical regions like the nucleocapsid, movement protein, glycoprotein precursor, and RNA-dependent RNA polymerase, offering a deeper understanding of the virus's genetic structure.

Analysis of Impatiens necrotic spot virus: Sequencing and Haplotype Network Construction

Sequencing Process. Direct sequencing was performed on all amplicons derived from the selected 20-sample subset, previously identified as positive for





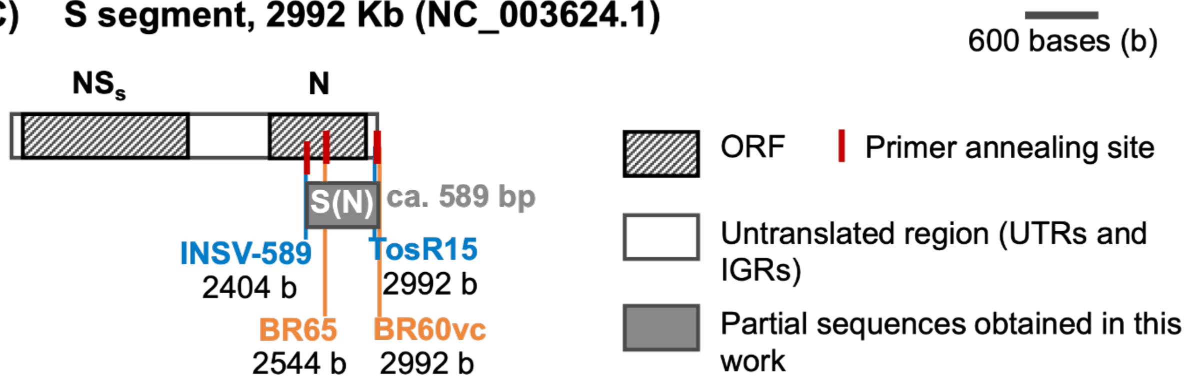


Figure 1. Representation of primer pairs annealing positions in reference to the complete genome of *Impatiens necrotic spot virus*; accession numbers in parenthesis for each genome segment. Overlapping among primers and the final partial sequences (gray rectangles) obtained herein with corresponding expected size (bp). Base pair values under each primer represent the positions of the first and last nucleotide of the corresponding amplicon in the reference genome.

INSV through ELISA. This sequencing, conducted in both forward and reverse directions, employed the identical primer pairs used in the PCR amplifications. Macrogen Inc. (Korea) was responsible for the purification and sequencing of these PCR products. The resulting sequence contigs were carefully assembled and meticulously edited using BioEdit software (version 7.2.5). The obtained sequences were archived in the GenBank database, a repository maintained by the National Institute of Health. Our approach to analyzing the phylogenetic relationships of INSV involved extracting sequences representative of various geographic locations and host plants from GenBank. We employed a specific search strategy focusing on "Impatiens necrotic spot", categorizing the sequences by their respective genome segments: S, M, and L. Initial phylogenetic trees were constructed for each of the amplified genome regions: S(N), M(NSM), M(G), and L(L). This phase included incorporating relevant sequences available from the GenBank. DNA substitution models were meticulously selected to fit our phylogenetic analyses, which we executed using the MEGA X software (Kumar *et al.,* 2018). However, the initial phylogenetic constructs did not reveal any definitive or robust clustering patterns. In our pursuit of a more granular understanding of INSV diversity at a global scale, we aligned the S(N) sequences from our study with 81 INSV sequences retrieved from the GenBank, focusing on a 261 bp segment of the N ORF. This alignment was accomplished using the MUSCLE algorithm and was refined by trimming sequence overhangs to ensure uniformity. Using the DnaSP6 software (Rozas *et al.,* 2017), we conducted a thorough haplotype determination and population analysis on this dataset, encompassing 79 isolates (excluding two due to missing geographical data). The sequences were thoughtfully categorized by their countries or regions for a detailed analysis. To visualize the genetic relationships and variations among these INSV isolates, we constructed a haplotype network. This was achieved using the PopART software (Population Analysis with Reticulate Trees, http://popart.otago.ac.nz), applying the TCS network inference method (Clement *et al.,* 2000) to map the intricate connections between the various haplotypes. In a subsequent, more detailed analysis, we aligned and concatenated different regions of the INSV genome that were successfully amplified and sequenced from the same sample. This analysis included twelve samples from Costa Rica (where sequence data for all four targeted regions were obtained) and five international INSV samples with available complete genomes. The comprehensive alignment, spanning 2,945 positions, was subjected to a Maximum Likelihood analysis using a Tamura 3-parameter model with a gamma-distributed rate variation (+G), and this process involved 2,000 permutations in the MEGA X software. The analyses were conducted for all genome segments, both with and without the inclusion of Tomato spotted wilt virus (TSWV) as an outgroup, utilizing its reference sequences (S: NC_002051; M: NC_002050; and L: NC_002052) for comparative purposes.

Results

ELISA Analyses and Observed Symptoms. The ELISA tests identified INSV and IYSV in the samples, with INSV being relatively common in Costa Rica (58 out of 295 samples). Notably, TSWV and the serogroup comprising GRSV and TCSV were not detected. INSV was found in 12 out of 39 plant species tested, including pepper (*Capsicum annuum*), onion (*Allium cepa*), tomato (*Solanum lycopersicum*), and irises (*Iris* sp.), which had the highest number of positive samples (Table 2). Common symptoms associated with INSV included chlorotic or necrotic rings and spots, and chlorotic ring patterns (Figure 2). IYSV was exclusively detected in 10 onion leaf samples.

Longitudinal Analysis of TSWV and INSV Occurrence. Our investigation into the historical presence of *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus* (INSV) in Costa Rica was motivated by the absence of TSWV in our recent sample studies and the fact that the only documented occurrences of TSWV in the country were reported in 1990 (Rivera *et al.,* 1990) and 1991 (Hsu and Lawson, 1991). To delve deeper into this matter, we reviewed data from the diagnostic-clinic services records spanning from 2000 to July 2023.These records predominantly consisted of ornamental plants intended for exportation or importation but also included horticultural samples from growers ranging from small to large scale. A total of 1027 samples were analyzed for TSWV and 1889 for INSV. Out of these, 43 samples (4.2 %) tested positive for TSWV, and 290 samples (15.4 %) for INSV, as determined by DAS-ELISA using commercial antibodies (Table 3). Notably, between 2008 and 2010, the annual number of INSV-positive samples exceeded 50. In contrast, the count of TSWV-positive samples remained below ten each year. Post-2015, the frequency of detecting both viruses significantly decreased. Over the 23.5 years covered in the analysis, TSWV was detected in only 11 years, whereas INSV was detected in 17 years, as depicted in Figure 3. This trend underscores the changing patterns of virus prevalence and highlights the need for ongoing surveillance.

RT-PCR Confirmation of INSV Occurrence. RT-PCR analyses substantiated the presence of INSV in nine different plant taxa: *Antirrhinum majus, Capsicum annuum, Hippeastrum, Impatiens, Iris, Ocimum basilicum, Plectranthus scuttellarioides, Solanum lycopersicum*, and the artificially created orchid hybrid Bakerara. All plant species that tested positive for INSV via ELISA also yielded positive results in RT-PCR tests using at least two different primer pairs, followed by sequencing (as shown in Figure 1 and Table 4). Exceptions to this included *Browallia* sp., *Eustoma* sp., and onion samples. The Browallia and Eustoma plants, unfortunately, wilted and

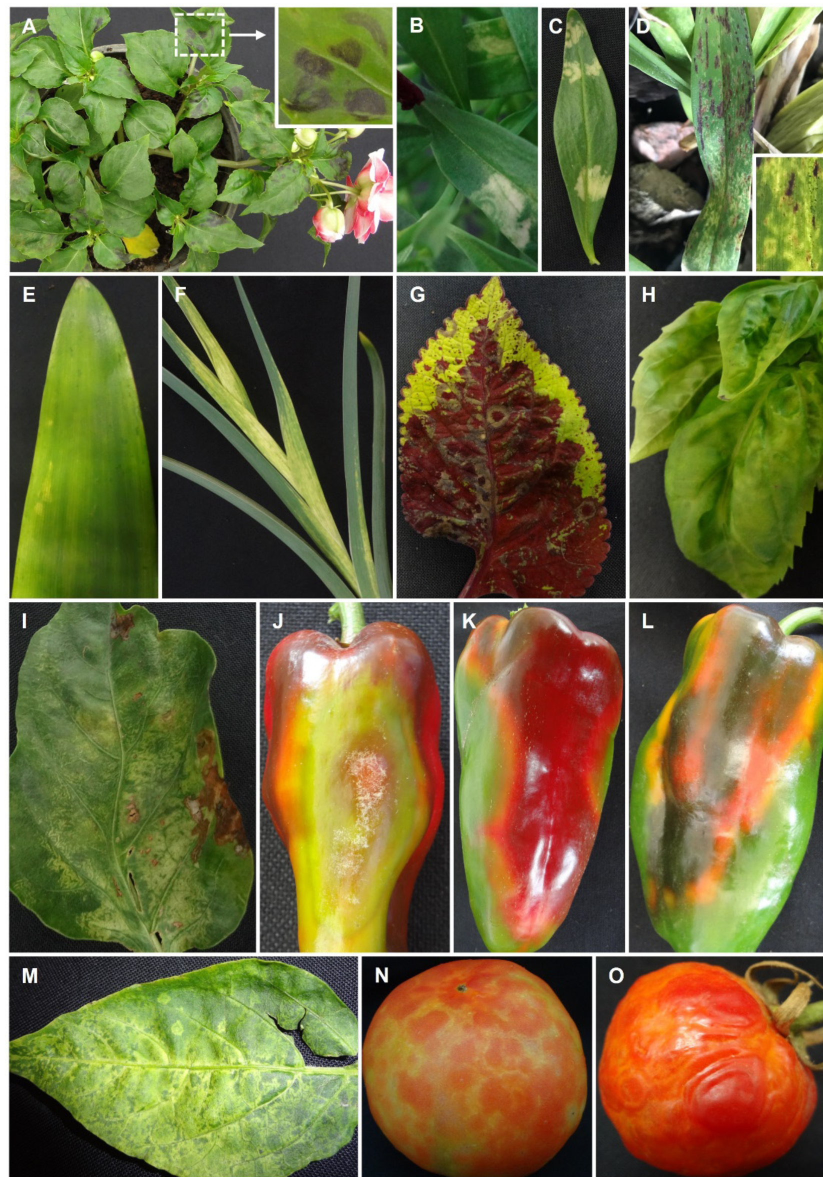


Figure 2. Diverse Symptoms of *Impatiens necrotic spot virus* (INSV) in Multiple Hosts Confirmed by ELISA and RT-PCR/Sequencing. A: Necrotic concentric rings on Impatiens (*Impatiens sp.)* T031. B, C: Chlorotic rings and spots on Snapdragons (*Antirrhinum majus*) T057. D: Chlorotic and necrotic striate on orchid leaves T024. E, F: Chlorotic mosaic on Amaryllis (*Hippeastrum* sp.) T161 and Iris (*Iris* sp.) T184. G: Necrotic concentric rings on Coleus (*Plectranthus scuttellarioides*) T157. H: Chlorotic patterns on Basil (*Ocimum basilicum*) T156. I, M: Chlorotic concentric ring patterns on Sweet Pepper (*Capsicum annuum*) leaves T250 and T092. J, K, L: Uneven ripening, ring patterns, and deformation on Sweet Pepper fruits T092, T107, T094. N, O: Chlorotic patterns, rings, and uneven surface on Tomato (*Solanum lycopersicum*) fruits T263 and T303.

Table 3. Number of total and positive samples per plant species tested by ELISA to detect three orthotospoviruses in Costa Rica.

Plant host (common name)	Sample number	Nº positive samples[y]			
		INSV	IYSV	TSWV	GRSV/ TCSV
Amaryllidaceae					
Allium cepa (onion)	38	6	10	0	0
Allium sativum (garlic)	2	0	0	0	0
Hippeastrum sp.("amaryllis")	1	1	0	0	0
Tulbaghia violaceae (society garlic)	1	0	0	0	0
Anacardiaceae					
Spondias purpurea (jocote)	1	0	0	0	0
Apocynaceae					
Mandevilla sp. (dipladenia)	1	0	0	0	0
Trachelospermum jasminoides (star jasmine)	1	0	0	0	0
Cataranthus roseus (periwinkle)	4	0	0	0	0
Asparagaceae					
Ornithogalum sp. (star of Bethlehem)	1	0	0	0	0
Asteraceae					
Lactuca sativa (lettuce)	6	0	0	0	0
Balsaminaceae					
Impatiens sp. (impatiens)	7	3	0	0	0
Caricaceae					
Carica papaya (papaya)	5	0	0	0	0
Cucurbitaceae					
Citrullus lanatus (watermelon)	2	0	0	0	0
Cucumis melo (muskmelon)	13	0	0	0	0
Cucumis sativus (cucumber)	1	0	0	0	0
Cucurbita pepo (pumpkin, zucchini)	2	0	0	0	0
Sechium edule (chayote)	1	0	0	0	0
Sechium tacaco (tacaco)	1	0	0	0	0
Fabaceae					
Arachis pintoi (pinto peanut)	3	0	0	0	0
Phaseolus vulgaris (black bean)	4	0	0	0	0
Gentianaceae					
Eustoma sp. (lisianthus)	3	3	0	0	0
Iridaceae					
Iris sp. (iris)	4	4	0	0	0
Lamiaceae					
Ocimum basilicum (basil)	1	1	0	0	0
Plectranthus scuttellarioides (coleus)	2	2	0	0	0
Liliaceae					
Lilium sp. (lilies)	2	0	0	0	0
Malvaceae					
Gossypium hirsutum (cotton)	1	0	0	0	0
Hibiscus sp. (hibiscus)	4	0	0	0	0
Orchidaceae					
Several species and hybrids[z] (orchids)	45	1	0	0	0
Oxalidaceae					
Oxalis sp.	2	0	0	0	0

Table 3. Continue...

Plant host (common name)	Sample number	Nº positive samples[y]			
		INSV	IYSV	TSWV	GRSV/ TCSV
Plantaginaceae					
Antirrhinum majus (snapdragon)	2	2	0	0	0
Rosaceae					
Fragaria x ananassa (strawberry)	2	0	0	0	0
Solanaceae					
Browallia sp. (bush violet)	2	1	0	0	0
Calibrachoa sp. (million bells)	4	0	0	0	0
Capsicum annuum (sweet pepper)	68	30	0	0	0
Petunia sp. (petunia)	14	0	0	0	0
Solanum lycopersicum (tomato)	37	4	0	0	0
Solanum melongena (eggplant)	1	0	0	0	0
Solanum quitoense (naranjilla, lulo)	1	0	0	0	0
Solanum tuberosum (potato)	4	0	0	0	0
Zingiberaceae					
Hedychium sp.	1	0	0	0	0
Total	**295**	**58**	**10**	**0**	**0**

[y] Samples were tested to detect: *Impatiens necrotic spot virus* (INSV), *Iris yellow spot virus* (IYSV), *Tomato spotted wilt virus* (TSWV) or the serogroup *Groundnut ring spot virus/Tomato chlorosis spot virus* (GRST/TCSV)
[z] Orchid samples included several different genera and hybrids; therefore, those are listed together as family Orchidaceae. The positive sample corresponded to a plant of the hybrid genus *Bakerara*.

died in our greenhouse before we could collect tissue samples for RNA extraction, preventing their testing by RT-PCR. Additionally, onion samples were not tested by RT-PCR as the presence of IYSV in these samples had already been confirmed in a previous study (Montero-Astúa *et al.,* 2017). In total, we successfully obtained and edited 72 final partial sequences or contigs. These included 18 sequences each for the nucleocapsid and movement protein genes, 20 for the glycoprotein precursor gene, and 16 for the viral RNA-dependent RNA polymerase (RdRp) gene. All of these sequences have been submitted to GenBank and are documented in Table 4.

Haplotype Network Analysis. In the initial phylogenetic analysis of the S(N), M(NSM), M(G), and L(L) sequences of INSV, including sequences from other countries, we noticed a lack of well-defined clusters (data not shown). Consequently, we shifted our focus to a haplotype analysis of the S(N) sequence dataset. This dataset comprised a 261 bp segment from the nucleocapsid ORF of INSV isolates globally available in the GenBank, along with the 18 sequences we obtained in this study (Figure 4). In our haplotype network analysis, we identified four distinct haplotypes of the partial nucleocapsid ORF sequence within Costa Rica. Among

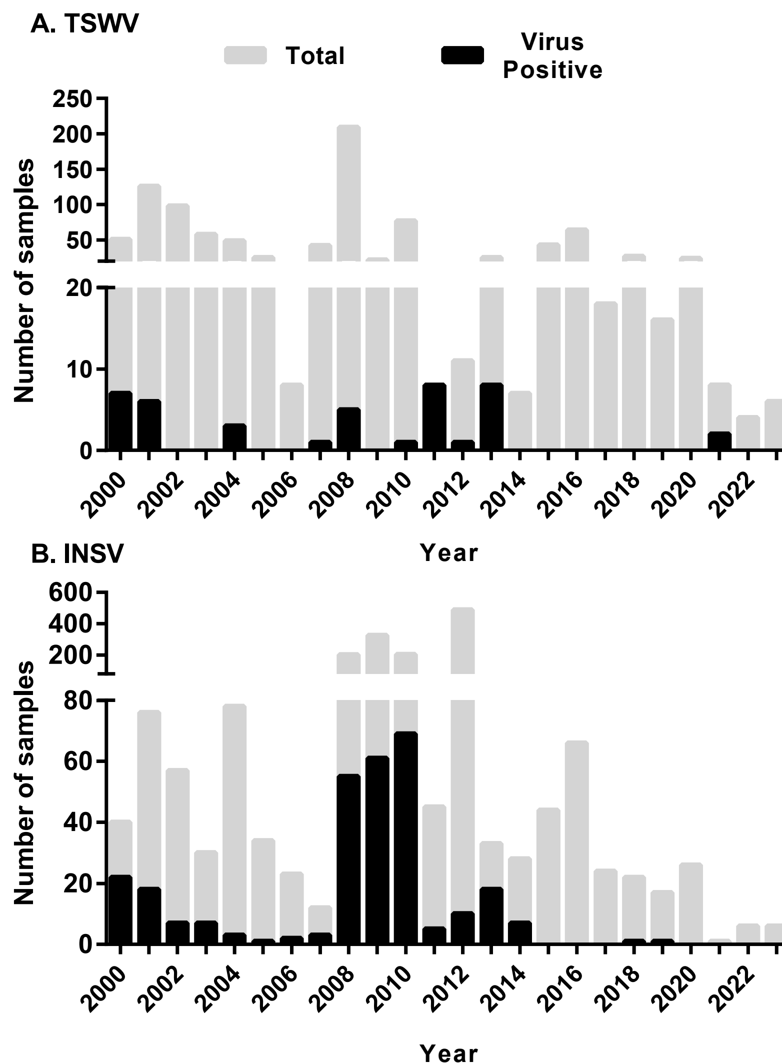


Figure 3. Long-Term Detection of impatiens necrotic spot and tomato spotted wilt viruses by ELISA. This graph presents the frequency of detection for both Impatiens necrotic spot virus and Tomato spotted wilt virus over a 23.5-year period (2000 to July 2023). The data, derived from samples submitted to the diagnostic services at the Cellular and Molecular Biology Research Center, University of Costa Rica, illustrate the temporal trends and occurrence patterns of these viruses in Costa Rica.

these, one haplotype emerged as predominant in the country, which interestingly is also found in China, Japan, Europe, and Korea. The other two haplotypes, although less prevalent in Costa Rica, are more closely related to a major haplotype observed in Europe, Japan, New Zealand, and the USA. Further, we conducted a concatenated analysis of four genome regions (S, M, and L genome segments) that we amplified and sequenced. This analysis included 12 samples from Costa Rica and five INSV

Table 4. Partial nucleotide sequences and virus identity determined for a subset of INSV ELISA-positive samples.

Plant host	ID	Site[w], province	Sequence[x] identification[y]			
			S(N)	M(NS$_M$)	M(G)	L(L)
Antirrhinum majus	T057	D, Cartago	MH687991	MH673625	MH673643	MH487473
Capsicum annuum	T092	N, Alajuela	MH687992	MH673626	MH673644	MH487474
	T093	M, Alajuela	MH687993	MH673627	MH673645	MH487475
	T094	M, Alajuela	MH687994	MH673628	MH673646	MH487476
	T096	M, Alajuela	MH687995	MH673629	MH673647	MH487477
	T107	N, Alajuela	MH687996	MH673630	MH673648	MH487479
	T109	N, Alajuela	MH687997	MH673631	MH673649	MH487480
	T245	II, Cartago	MH688003	MH673638	MH673657	MH487487
	T250	II, Cartago	MH688004	MH673639	MH673658	MH487488
	T251	II, Cartago	MH688005	MH673640	MH673659	MH487489
Hippeastrum sp.	T161	D, Cartago	MH688001	MH673635	MH673653	MH487483
Impatiens sp.	T031	C, Alajuela	MH687990	MH673624	MH673642	MH487472
Iris sp.	T184	CC, Cartago	---[z]	MH673636	MH673654	MH487484
	T186	CC, Cartago	---[z]	MH673637	MH673655	MH487486
Ocimun basilicum	T156	D, Cartago	MH687998	MH673632	MH673650	---[z]
Orchid	T024	B, Alajuela	MH687989	MH673623	MH673641	---[z]
Plectranthus	T157	D, Cartago	MH687999	MH673633	MH673651	MH487481
scuttellarioides	T158	D, Cartago	MH688000	MH673634	MH673652	MH487482
Solanum lycopersicum	T263	O, San José	MH688006	---[z]	MH673660	---[z]
	T303	O, San José	MH688007	---[z]	MH673661	---[z]
Total (*n*)	20	8, 3	18	18	20	16

[w] Different letter codes distinguish independent fields, greenhouses, parks, or gardens.

[x] Sequences corresponding to partial fragments of the S, M and L genome segments of orthotospoviruses obtained from one or several primer pairs (contig) as shown in Figure 1. **S(N)**, partial sequence of the nucleocapsid ORF and 3'-UTR regions of the S genome segment. **M(NS$_M$)**, partial sequence of the movement protein ORF (NS$_M$). **M(G)**, partial sequence of the glycoprotein precursor ORF. And **L(L)**, an internal region of the polymerase (RdRp) ORF in the L genome segment.

[y] Plant virus species determined by the first hit BLAST result (GenBank, National Center for Biotechnology Information, U.S.A.) and percentage of identity >97%.

[z] ---, no sequence available because: (i) there was no amplification in RT-PCR; alternatively, (ii) an amplicon was generated but the sequencing reaction failed (one or both primers), or yielded only plant host sequence (nonspecific amplification).

genomes available in GenBank (illustrated in Figure 5). The results showed that the Costa Rican INSV samples clustered together, distinctly separate from the reference sequences. Notably, the sub-cluster structures of the Costa Rican samples generally aligned with their geographical sites of origin. This finding indicated a greater diversity within the Costa Rican INSV population compared to what was suggested by the haplotype network. In the phylogenetic tree, the branches and nodes clearly delineated the differences among the 12 Costa Rican isolates included in our study (Figure 5). This variation in genetic diversity highlights the complex nature of INSV spread and evolution within different geographic regions.

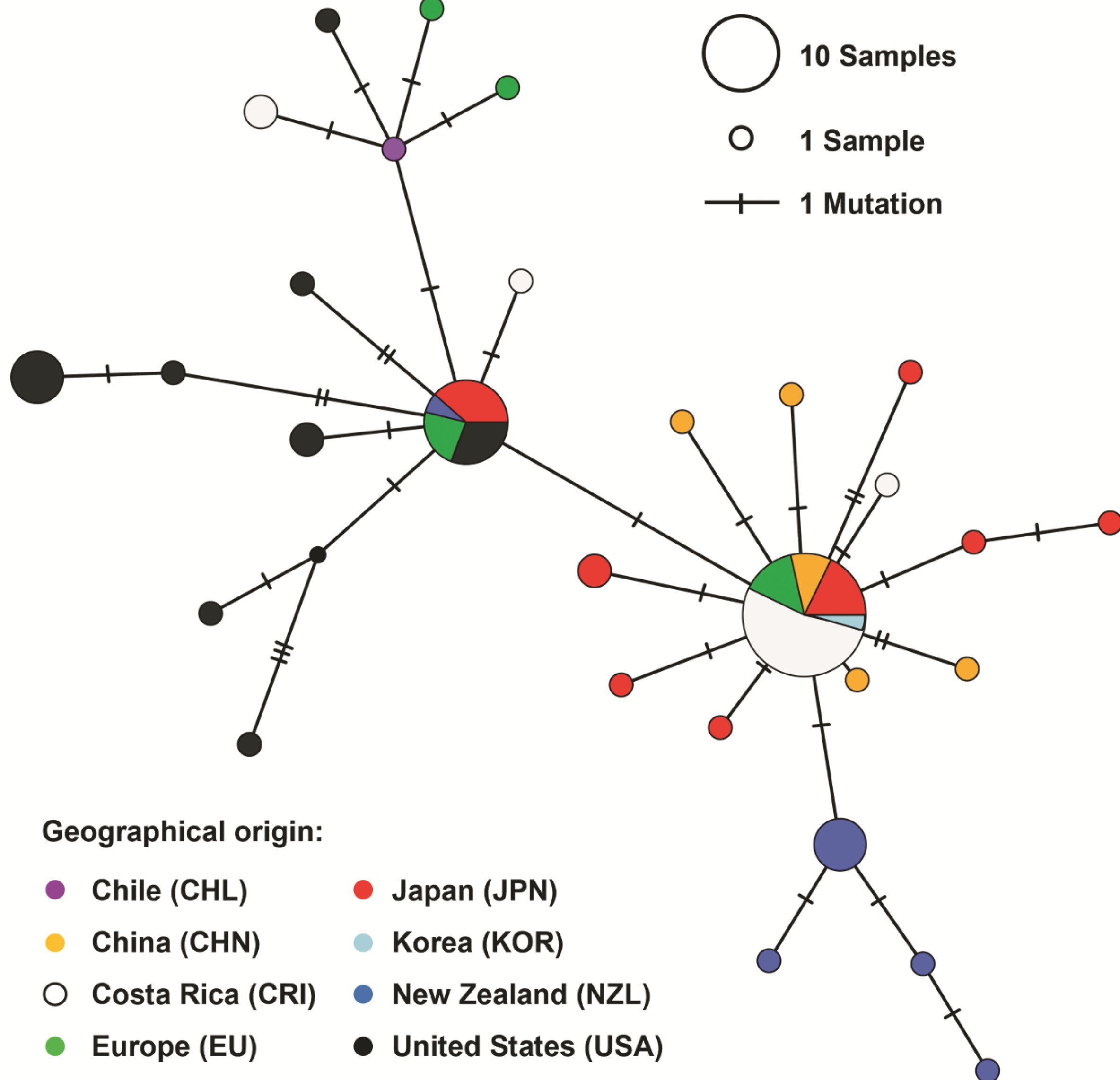


Figure 4. Haplotype Network Graph of Impatiens Necrotic Spot Virus. This graph is a haplotype network inferred using the TCS method, based on a 261 bp sequence alignment of the nucleocapsid protein ORF (S[N] segment) of Impatiens necrotic spot virus. The network includes data from 81 isolates, visually representing the genetic relationships and diversity among the different haplotypes of the virus.

Discussion

In our study, we focused on the historical presence of TSWV and INSV in Costa Rica, prompted by the absence of TSWV in our recent samples and only sporadic reports of TSWV dating back to 1990 and 1991. Our analysis of records from the

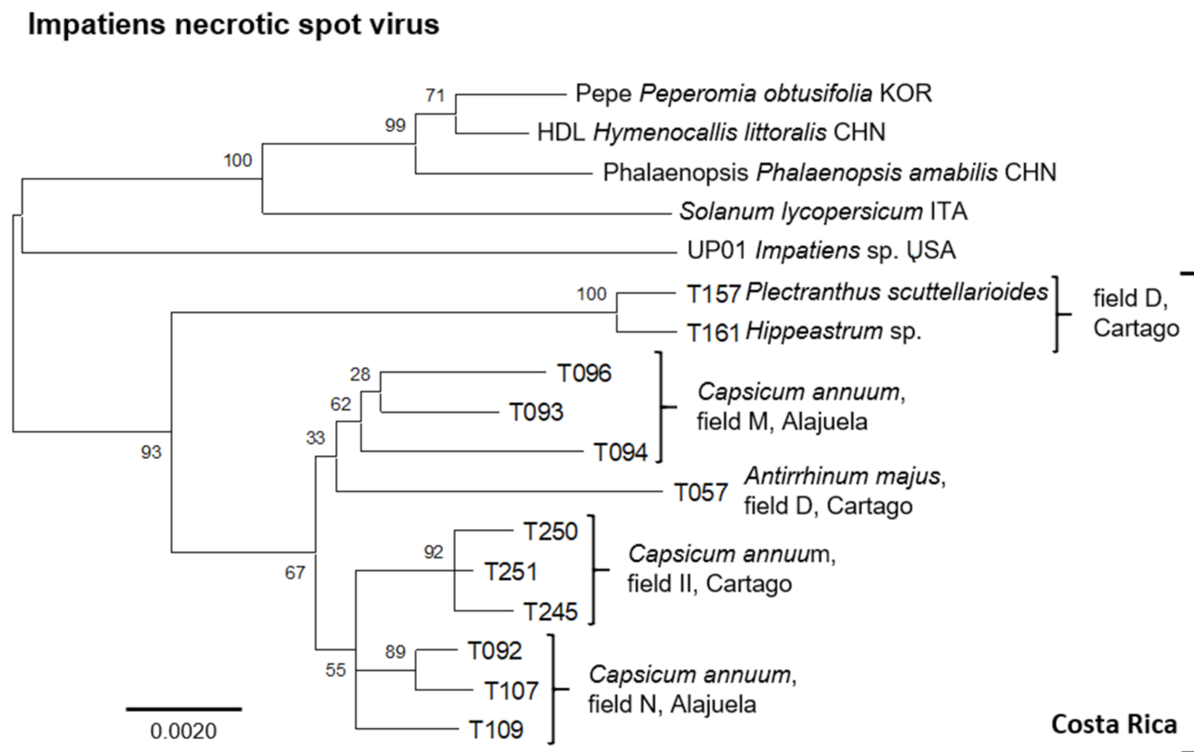


Figure 5. Comprehensive phylogenetic analysis of impatiens necrotic spot virus isolates. This figure illustrates a phylogenetic tree constructed from an alignment of concatenated sequences (2945 positions) encompassing regions of the nucleocapsid [S(N)], glycoprotein precursor [M(G)], movement protein [M(NSM)], and viral polymerase [L(L)] ORFs. The tree includes isolates from various countries, identified by ISO 3166-1 three-letter codes: CHN (China), ITA (Italy), KOR (South Korea), and USA (United States of America). For Costa Rican isolates, additional distinct letter codes in parenthesis signify separate geographic locations. The analysis, executed in MEGA X, utilized the Maximum Likelihood method with a Tamura-3-parameter model and a gamma-distributed rate of variation in nucleotides (+G), involving 2000 permutations. The scale bar indicates the number of nucleotide substitutions per site.

diagnostic clinic services, covering the period from 2000 to July 2023, revealed a low occurrence of TSWV, with alternating periods devoid of detection. This pattern aligns with global trends suggesting cyclical fluctuations in TSWV occurrences, as indicated by Tentchev *et al.* (2011) and further discussed by Kaye *et al*. (2011) in relation to the TSWV population dynamics in peanut crops in North Carolina and Virginia. These dynamics may be influenced by various factors, including vector biology, thrips feeding preferences, and previously implemented TSWV-targeted control measures in Costa Rica. The recent shifts in vector populations and competition among whitefly species, as noted in studies by Can-Vargas *et al*. (2020) and Valverde-Méndez *et al*. (2023), could also contribute to these observed trends.

Notably, our survey did not detect the orthotospovirus serogroup GRSV/TCSV, suggesting its absence in the continental Caribbean region of Central America. This is despite its prevalence in the Caribbean islands. The use of degenerate *orthotospovirus* primers in our study, which did not identify viruses other than INSV, indicates a possible lack of diversity among orthotospoviruses in Costa Rica. Our survey, conducted between 2013 and 2014, emphasizes the need for ongoing surveillance to monitor potential introductions of new orthotospovirus species.

Our findings establish INSV as a prevalent orthotospovirus in Costa Rica, in contrast to its apparent absence in other Central American countries. This discrepancy likely reflects a gap in regional orthotospovirus research rather than an actual absence of the virus. Previous studies have identified two distinct clades of INSV isolates, the Western Hemisphere and Asian clades. Our haplotype network analysis, based on a partial nucleocapsid sequence, suggests the presence of two main haplotypes and various secondary nodes, with most Costa Rican sequences aligning with the putative Asian clade. This observation indicates at least two separate introductions of INSV into Costa Rica. Additionally, our results suggest a geographical clustering of INSV isolates within Costa Rica, with evidence of genetic differentiation based on the site of origin. This is in line with findings from previous studies, which also indicated a tendency for INSV isolates to cluster geographically and reported limited genetic diversity within local INSV populations.

Our study underscores the need for further research to accurately assess the genetic diversity of the INSV population in Costa Rica. Such efforts are crucial for understanding the epidemiology of orthotospoviruses in the region and for developing effective management strategies.

Conclusions

Our comprehensive survey conducted in 2013 and 2014 across Costa Rica has yielded significant insights into the prevalence and diversity of orthotospoviruses in the region. Impatiens necrotic spot virus (INSV) was detected in a significant number of samples (58 out of 295) tested via ELISA. The virus exhibited a wide host range, affecting 13 different taxa including *Allium cepa, Capsicum annuum, and Solanum lycopersicum,* among others. Conversely, IYSV was only identified in ten onion samples. Notably, TSWV, GRSV, and TCSV were not detected in this study. However, historical data confirm the presence of TSWV in Costa Rica, with a noted decrease in detection requests and occurrences over time.

The analysis based on a fragment of the nucleocapsid sequence indicated low diversity of INSV in Costa Rica, with only four haplotypes identified among the 20 samples analyzed. A more in-depth analysis, including concatenated regions of the

S, M, and L genome segments, suggested a relatively higher variability of INSV within the country. This discrepancy underscores the need for further sampling and analysis to accurately determine the genetic diversity of INSV in Costa Rica.

Our data suggest the presence of two lineages of INSV in Costa Rica - the Asian and Western Hemisphere lineages. This hypothesis aligns with the global distribution patterns of INSV and indicates multiple introduction events into the region. Given the dynamic nature of international trade and human movement, an updated and more extensive survey across the whole country is essential to understand the current status of orthotospoviruses in Costa Rica. Furthermore, there is a critical need for comprehensive research throughout Central America. This research should focus on symptom descriptions, susceptible host lists, and identification of thrips populations involved in virus transmission, to understand the epidemiology of orthotospoviruses in the region. Acquiring sequence data will be crucial for understanding population structures and identifying potential high-risk strains, such as those capable of breaking resistance. This information is vital for developing strategies to control the introduction and spread of specific viral strains and species in the region. Effective management strategies, including customs inspections, quarantine measures, and regional collaboration, are imperative to mitigate the impact of these viruses on agriculture in Central America.

In conclusion, our study highlights the importance of continuous monitoring, research, and regional cooperation in managing orthotospoviruses, a critical step towards ensuring agricultural sustainability and productivity in Central America.

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