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Papaya ringspot virus W infecting *Luffa aegyptiaca* in Cuba

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Abstract *Luffa aegyptiaca* (sponge gourd) plants showing severe leaf mosaic and deformation symptoms were observed in Villa Clara, Cuba. Electron microscopy observations of leaf dip preparations revealed flexuous filamentous particles, which were identified as *Papaya ringspot virus* (PRSV) by ELISA test. PRSV was mechanically transmitted to healthy *Cucurbita moschata* plants. Sequence analysis of the RT-PCR product obtained from the capsid protein gene showed highest identity with other PRSV isolates from United States of America, Australia, Venezuela, Brazil, India and Cuba, ranging between 93.2–96.5% and 93.4–98.1% for nucleotide and amino acid sequences, respectively. Phylogenetic analysis showed two main clusters. Cluster I included isolates from the Americas-Australia group and India, including sponge gourd isolate, while the cluster II included isolates from China, Thailand and Taiwan. To our knowledge, this is the first report of PRSV-W infecting sponge gourd in Cuba.

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Papaya ringspot virus (PRSV) is a worldwide virus that infects mainly cucurbits and papaya plants with devastating consequences. PRSV is classified into two serologically indistinguishable strains with distinct biological characteristics. The watermelon strain (PRSV-W) infects cucurbits but not papaya, and the papaya strain (PRSV-P) is capable of systemically infecting species in both Cucurbitaceae and Caricaceae families (Tripathi et al. 2008).

In March 2015, plants of sponge gourd (*Luffa aegyptiaca*) showing severe mosaic and leaf deformation symptoms were observed (Fig. 1) and collected from Villa Clara province, Cuba. These symptoms were similar to those induced by PRSV in other cucurbits (Romay et al. 2014a). The causal agent was identified using electron microscopy, serological analyses, experimental transmission studies, reverse transcription-polymerase chain reaction (RT-PCR) and sequencing.

Symptomatic sponge gourd leaf tissues were analyzed by transmission electron microscopy; leaf-dip preparations were made with borate buffer 0.05 M pH 7 and stained with 2% uranyl acetate (Sigma-Aldrich, USA) (Kitajima and Nome 1999). The observations revealed the presence of typical flexuous potyvirus particles of ca. 800 × 12 nm (data not shown). Moreover, leaf extracts obtained from macerated symptomatic leaves were positive by ELISA when a specific PRSV antiserum (Agdia, USA) was used.

Leaves from naturally infected sponge gourd plants were analyzed by mechanical inoculation onto healthy *Carica papaya* (five) and *Cucurbita moschata* (five) plants. Plants were dusted with silicon carbide 600 mesh on the third and fourth youngest leaves and gently rubbed with sponge gourd

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Fig. 1 Symptoms of vein clearing, mosaic and leaf deformation caused by a Cuban *Papaya ringspot virus* W isolate on naturally infected sponge gourd plant

leaf extract in a 1:10 (*w*/*v*) dilution, containing 0.01 M potassium phosphate buffer (pH 7.0) and 0.1% sodium sulphite. Plants of each species were rubbed with inoculation buffer as control. Plants of *C. moschata* showed severe symptoms (data not shown); however, no symptoms were observed on papaya, suggesting that PRSV infecting sponge gourd belongs to the W strain. In the tested plants (*C. papaya*, *C. moschata* and *L. aegyptiaca*), the presence or absence of PRSV was assayed by RT-PCR.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. Primers designed to amplify the coat protein (CP) gene of PRSV were used (Cabrera Mederos et al. 2016). The reactions to amplify the CP were performed in two steps. Reverse transcription was performed using M-MLV enzyme (Promega, USA), which was incubated for 4 min at 99 °C, followed by 60 min of incubation at 42 °C. PCR was performed using 2 μl of cDNA, 1 U of Kapa Hiffi DNA polymerase enzyme (Kapa Biosystems, USA), 300 μM dNTPs each and 0.3 μM of each primers in a final reaction volume of 50 μl. The PCR conditions included an initial incubation for 2 min at 94 °C, followed by 34 cycles of denaturation at 98 °C for 30 s, annealing at 58 °C for 45 s, extension for 1 min at 72 °C and a final extension at 72 °C for 10 min.

The obtained RT-PCR amplicons of the expected size (850 bp) (data not shown) were purified and then directly sequenced in both directions by Macrogen Inc., Seoul, Korea. To search for related sequences, a preliminary sequence analysis was performed with the NCBI blast tool. Blast analysis of the sequenced fragment, which was deposited in GenBank (Accession number KX385117), revealed highest nucleotide and amino acid identities with PRSV sequences from cucurbit plants from the Americas and Australia. PRSV CP sequences of different origins were selected from GenBank database for further analysis. Sequence analysis was performed using MEGA 7.0 software (Kumar et al. 2016), and sequence alignment of PRSV isolates was carried out using MUSCLE (Edgar 2004). After sequence trimming, a 819 bp fragment was used to estimate the molecular variability of PRSV.

The analysis of multiple sequences alignment revealed that sponge gourd PRSV isolate shared nucleotide identities between 93.2–96.5% with sequences from United States of America, Venezuela, Australia, Brazil, India and Cuba, whereas identities with China, Thailand and Taiwan isolates were 89.0–90.5% (Table 1). The CP nucleotide identity values observed from the comparison of the Cuban sponge gourd isolate with other PRSV isolates were above 89%, which is higher than the current potyvirus species demarcation criterion

Table 1 Nucleotide (nt) and amino acid (aa) sequence identities (%) of a Cuban *Papaya ringspot virus* W isolate from *Luffa aegyptiaca* (GenBank accession number KX385117) with isolates from other geographical regions

GenBank accession number-Origin	Identities		Strain
	nt	aa	
JN132457-US	96.5	98.1	W
JN132441-US	96.3	98.1	W
JN132440-US	96.3	97.8	W
JN132451-US	96.2	98.1	W
JN132445-US	96.2	97.8	W
EF189736-VE	96.2	97.8	P
JN132447-US	96.0	97.0	W
S89893-AU	95.9	97.4	W
JN132461-US	95.4	98.1	W
KC345585-VE	95.3	97.8	W
KC345574-VE	95.1	97.8	W
KC345572-VE	94.9	97.8	W
JN979403-IN	94.8	98.1	W
KC748227-CU	94.8	95.2	P
AF196839-US	94.6	96.3	P
KC768854-CU	94.1	97.0	W
AY841757-CU	94.0	93.7	P
KC748229-CU	93.7	95.2	P
JN979405-IN	93.4	97.4	W
AF530088-BR	93.4	95.6	W
DQ374153-BR	93.4	95.6	W
KP019380-CU	93.4	93.4	W
AF344640-BR	93.4	95.6	P
AF344639-BR	93.2	95.6	P
AY027811-TW	90.5	95.2	W
AY010722-TH	90.1	94.8	W
DQ868880-CN	89.8	95.2	W
AY010718-TH	89.0	93.4	P

AU Australia, *BR* Brazil, *CN* China, *CU* Cuba, *IN* India, *TW* Taiwan, *TH* Thailand, *US* United States of America, *VE* Venezuela

(76%), set by the ICTV (Adams et al. 2005). Nucleotide diversity among Cuban PRSV-W isolates showed values ranging from 5.9 to 6.6%, whereas that variation among Cuban PRSV-P isolates was 2.7–3.5 (data not shown). PRSV is considered a highly variable virus species, with variability of the CP nucleotide among isolates of different countries ranging between 1.5% (Australia) and 10.1% (India) (Bateson et al. 2002; Fernández-Rodríguez et al. 2008).

The EK (glutamic acid and lysine) repeat boxes reported previously in the N-terminal region of the CP gene of PRSV (Silva-Rosales et al. 2000) were present in the sponge gourd PRSV-W isolate. The conserved DAG, WCIEN, and QMKAAA domains (Wei et al. 2007) were also present in the sponge gourd isolate, as well as in the other PRSV sequences analyzed (data not shown).

Phylogenetic relationships based on PRSV CP nucleotide sequences revealed two major clusters (Fig. 2). Cuban PRSV isolates clustered together with isolates from United States of America, Australia, Venezuela, India and Brazil, forming cluster I, whereas cluster II included other Asian isolates (China, Thailand and Taiwan). The grouping of PRSV by geographic region, which was observed in the maximum-likelihood tree, has been reported previously (Inoue-Nagata et al. 2007; Olarte-Castillo et al. 2011; Rodríguez-Martínez et al. 2014).

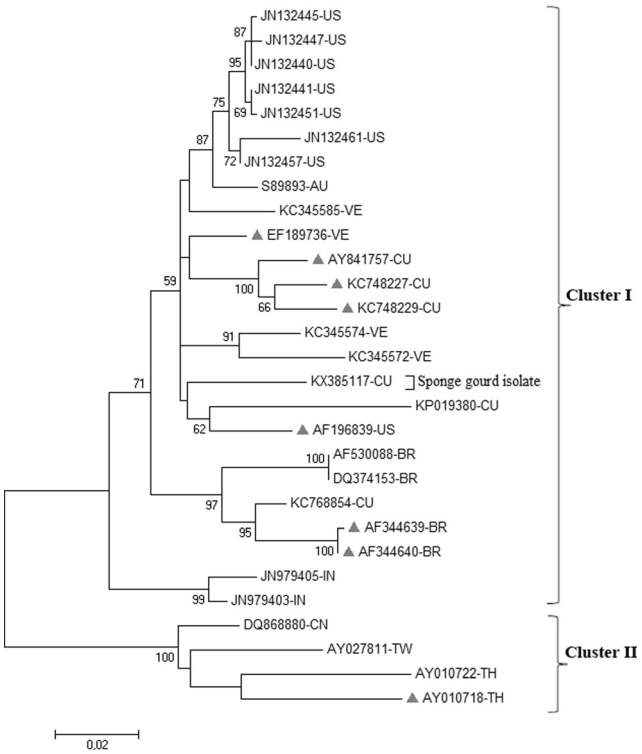

Fig. 2 Maximum-likelihood phylogenetic tree based on the partial coat protein nucleotide sequences of a sponge gourd PRSV-W isolate (KX385117) and other 28 PRSV-W and P isolates available at GenBank database. The tree was generated using MEGA7 with Tamura-Nei method (TN93 + G = 0.24). Bootstrap values (with 1000 replicates) greater than 50% are indicated at the tree nodes. For each isolate, the GenBank accession number and geographical origin are indicated. AU = Australia, BR = Brazil, CN = China, CU = Cuba, IN = India, TH = Thailand, TW = Taiwan, US = United States of America, VE = Venezuela

The association of American-Australian with some Indian PRSV isolates was previously reported (Silva-Rosales et al. 2000; Bateson et al. 2002). Furthermore, the phylogenetic analysis showed that PRSV-W and P isolates from different geographical locations grouped together, which could be associated with the PRSV dispersion from the Indian subcontinent to the Americas (Olarte-Castillo et al. 2011).

The sponge gourd Cuban isolate clustered together with a *Momordica charantia* Cuban PRSV-W isolate (GenBank accession number KP019380) (Cabrera Mederos et al. 2015), and other PRSV-W isolates from United States of America (Abdalla and Ali 2012), Australia and Venezuela (Bateson and Dale 1992; Romay et al. 2014b). However, the Cuban PRSV-W isolate of *Cucurbita pepo* was found to be grouped with the Brazilian PRSV-W isolates. Additional PRSV-W sequences from Cuba are required to perform compelling phylogenetic analyses because only three PRSV-W sequences were available from GenBank at the time of performing the present study.

In *L. aegyptiaca*, PRSV-W was previously reported in Asia region (Dahal et al. 1997; Verma et al. 2006); however, it has still not been detected in Latin America and the Caribbean islands. In Cuba, the PRSV-W strain has been found only in *M. charantia* (Cabrera Mederos et al. 2015) and *C. pepo* plants (Rodríguez-Martínez et al. 2015). However, to date, little is known in Cuba about the epidemiology of PRSV-W, which has been described as a major virus in cucurbits worldwide (Romay et al. 2014a). To our knowledge, this is the first report of PRSV-W on sponge gourd in Cuba, which is crucial for clarifying the variability, distribution and management of this disease in cucurbits.

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