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A cilevirus infects ornamental hibiscus in Hawaii

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Abstract The complete nucleotide sequence of a virus infecting ornamental hibiscus (*Hibiscus* sp.) in Hawaii with symptoms of green ringspots on senescing leaves was determined from double-stranded RNA isolated from symptomatic tissue. Excluding polyadenylated regions at the 3' termini, the bipartite RNA genome was 8748 and 5019 nt in length for RNA1 and RNA2, respectively. The genome organization was typical of a cilevirus: RNA1 encoded a large replication-associated protein with methyltransferase, protease, helicase and RNA-dependent RNA polymerase domains as well as a 29-kDa protein of unknown function. RNA2 possessed five open reading frames that potentially encoded proteins with molecular masses of 15, 7, 62, 32, and 24 kDa. The 32-kDa protein is homologous to 3A movement proteins of RNA viruses; the other proteins are of unknown function. A proteome comparison revealed that this virus was 92 % identical to citrus leprosis virus cytoplasmic type 2 (CiLV-C2), a recently characterized cilevirus infecting citrus with leprosis-like symptoms in Colombia. The high sequence similarity suggests that the virus described in this study could be a strain of CiLV-C2, but since the new genus *Cilevirus* does not have species demarcation criteria established at present, the classification of this virus infecting hibiscus is open to interpretation. This study represents the first documented case of a cilevirus established in the United States and provides insight into the diversity within the genus *Cilevirus*.

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The plant virus genus *Cilevirus* is a recently established taxon yet to be assigned to a virus family. *Citrus leprosis virus C* (CiLV-C) is the lone member of the genus and also represents the type species [6]. CiLV-C is associated with leprosis, an economically damaging disease of citrus in South and Central America that threatens major citrus-producing regions in Mexico and the United States [1]. CiLV-C has a short bacilliform virion approximately 50 by 130 nm in size that accumulates in the cytoplasm of infected cells [3] and is transmitted by mites of the genus *Brevipalpus* (Acari: Tenuipalpidae) [2]. It has a bipartite, positive-sense RNA genome. RNA1 is 8.7 kb in length and encodes a large replication-associated protein, which includes methyltransferase, protease, helicase, and RNA-dependent RNA polymerase domains, and a 29-kDa protein of unknown function. RNA2 is 5.0 kb in length and encodes proteins of 15 and 61 kDa, a 32-kDa putative movement protein (MP), and a 24-kDa protein. Both RNAs are polyadenylated at their 3' termini [7, 10]. Recently, a second putative cilevirus was detected in citrus with leprosis-like symptoms in Colombia. Designated CiLV-C2, this virus has similar characteristics to CiLV-C, although a 7-kDa protein encoded by RNA2 of CiLV-C2 has been proposed [11]. Kitajima et al. [5] have also documented

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numerous plant infections by agents that, based on cytology and vector, appear to have characteristics of cileviruses. The lack of molecular and serological data, however, has hindered progress in their classification.

In Hawaii, hibiscus (*Hibiscus* spp.) are popular ornamentals that include both native and introduced species and their hybrids. In November 2011, a hedgerow of *H. rosa-sinensis* in Honolulu was observed with green ringspot symptoms in senescing leaves (Fig. 1). Similar symptoms were subsequently observed in *H. rosa-sinensis* plants elsewhere on the island of Oahu. Total RNA was isolated from symptomatic tissues using a NucleoSpin® RNA Plant kit (Macherey-Nagel Inc., USA) following the manufacturer's directions and assayed by reverse transcription (RT)-PCR for hibiscus greenspot virus 2 (HGSV-2; formerly known as HGSV) [9]. All leaves tested negative for HGSV-2, suggesting the presence of a different pathogen. Double-stranded (ds)RNAs were isolated from symptomatic tissues as described [4]. Following electrophoresis, dsRNAs of approximately 9 and 5 kbp in size were observed (Fig. 1). These dsRNAs were used as templates for molecular cloning as described previously [8, 9].

RNA1 (GenBank Accession #KC626783) was found to be 8748 nt in length, excluding a polyadenylated 3' terminus, and encoded two open reading frames (ORFs) (Fig. 2a). The first, ORF1, encoded a 2507-aa, 285.3-kDa protein with methyltransferase, protease, helicase, and RNA-dependent RNA polymerase domains. The second, ORF2, encoded a 263-aa, 28.7-kDa protein of unknown function. The 5' and 3' termini of RNA1 were found to be 119 and 223 nt in length, respectively. RNA2 (GenBank accession no. KC626784) was found to be 5019 nt in length, excluding a polyadenylated 3' terminus, and encoded five ORFs (Fig. 2a). The first, p15, encoded a 130-aa, 15.0-kDa protein of unknown function. The second, p7, encoded a 62-aa, 7.0-kDa protein with transmembrane properties. The third, p62, encoded a 544-aa, 61.6-kDa protein of unknown function. The fourth encoded a 292-aa, 32.4-kDa protein with homology to the 3A MPs of RNA viruses. The fifth, p24, encoded a 206-aa, 23.7-kDa protein of unknown function, but it was found to be homologous to a similarly positioned ORF on RNA3 of HGSV-2. The 5' and 3' termini of RNA2 were 56 and 347 nt in length, respectively.

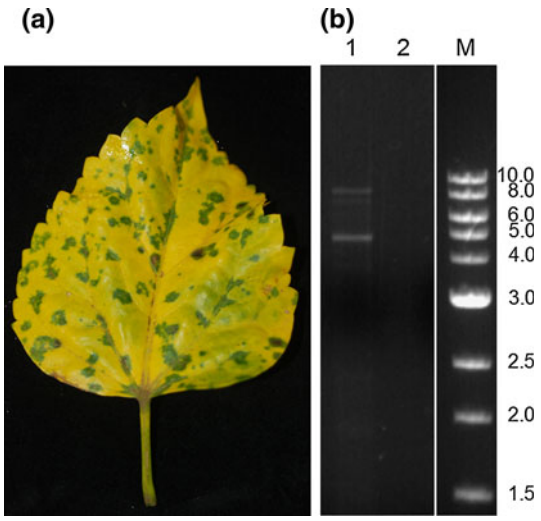

Fig. 1 **a** Green ringspot symptoms displayed in a senescing *Hibiscus rosa-sinensis* leaf infected with a cilevirus similar to citrus leprosis virus cytoplasmic type 2. **b** Double stranded RNAs isolated from symptomatic (lane 1) and asymptomatic (lane 2) leaves. Lane M is a 1-kb ladder (Mirus Bio Corp., USA) with fragment sizes indicated on the right in kilobase pairs

RNA1 and RNA2 were 86.2 and 80.0 % identical to RNA1 and RNA2 of CiLV-C2, respectively, indicating a close relationship between the two viruses (Fig. 2b). The amino acid sequence identities for ORF1 and ORF2 of RNA1 were 95 and 92 %, respectively, for these two viruses. The amino acid sequence identities for p15, p7, p62, MP, and p24 of RNA2 were found to be 78, 50, 86, 92, and 97 %, respectively, with the homologs of CiLV-C2. Overall, the amino acid identity between the proteomes of these two viruses was 92 %. Phylogenetic analysis also suggested a close relationship between these two viruses (Fig. 2c).

Despite the high sequence similarity between these two viruses, two distinguishing genomic features were observed. Roy et al. [11] identified an ORF in RNA2 of CiLV-C2 that encodes a 7-kDa transmembrane protein (p7). This ORF begins 160 nt downstream of p15 and terminates 645 nt upstream of p61. In the virus infecting hibiscus, however, the ORF encoding p7 is located much further downstream, beginning 515 nt downstream of p15 and terminating 420 nt upstream of p62 (Fig. 2a). In addition, a portion of the sequence encoding p7 is repeated upstream in a small ORF that has only weak transmembrane properties (data not shown). Overall, it appears that the region between the ORFs encoding p15 and p61/p62 is highly variable in cileviruses, an observation supported by the low sequence similarity in this region (Fig. 2b). Interestingly, the p7 ORF is absent in the genome of CiLV-C, suggesting that it is not a requirement for infecting citrus. The second distinguishing feature was the 5' terminus of RNA2. CiLV-C2 appears to possess 45 nt that are absent in the hibiscus-infecting virus (Fig. 2d).

Due to the current lack of characterized cileviruses, guidelines for species demarcation within this genus have yet to be established [6]. As such, the classification of the cilevirus infecting hibiscus described in this study as either a strain of CiLV-C2 or a member of a novel species is open to interpretation. Regardless of its eventual designation, the

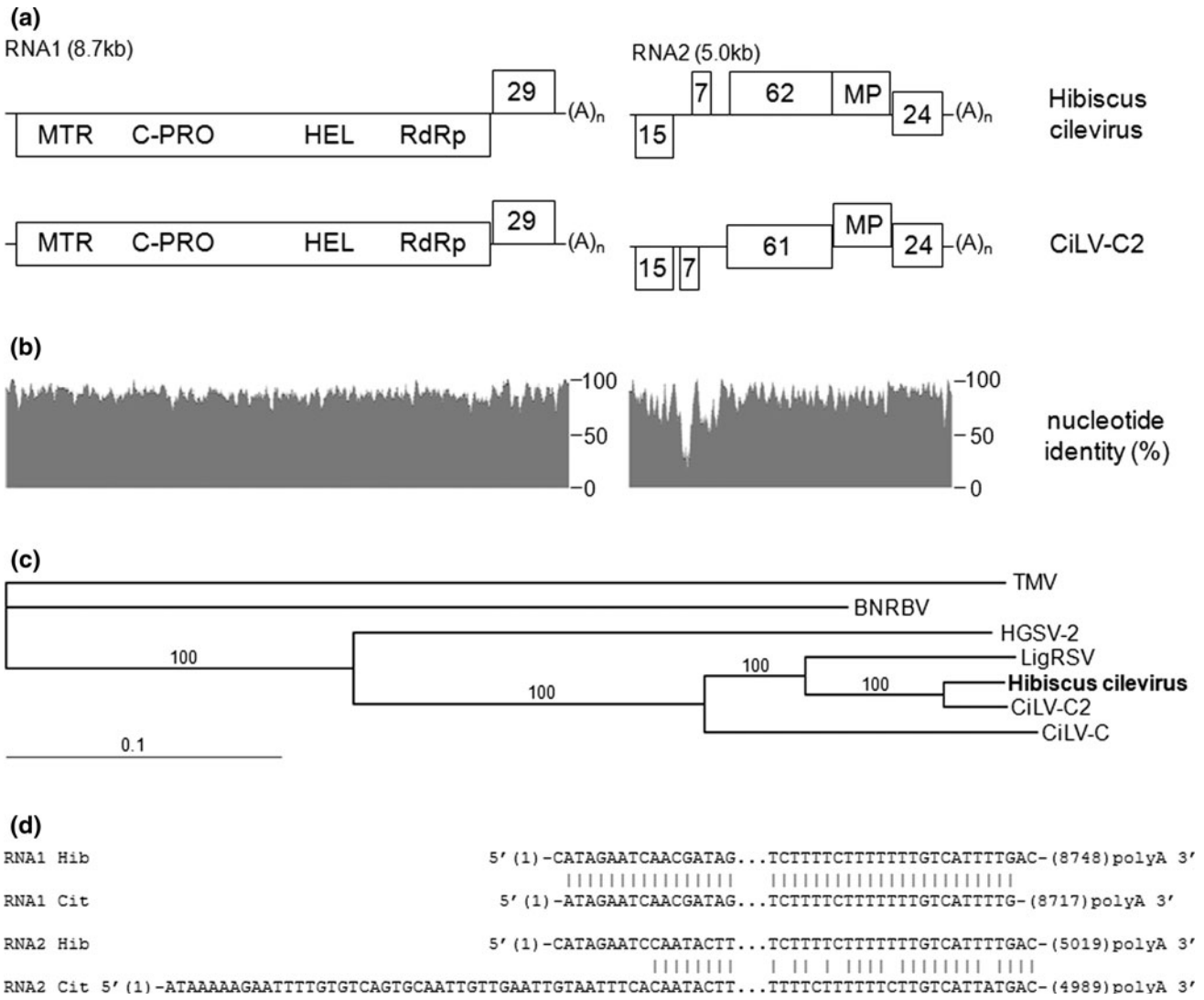

Fig. 2 **a** Genomic organization of the cilevirus infecting hibiscus and citrus leprosis virus cytoplasmic type 2 (CiLV-C2). Boxes represent putative open reading frames, with numbers indicating the approximate size of their protein products in kilodaltons. MTR, methyltransferase; C-PRO, cysteine protease; HEL, helicase; RdRp, RNA-dependent RNA polymerase; MP, movement protein; $(A)_n$, polyadenylated region. **b** Graphical representation of nucleotide sequence identity between the two viruses in **a**. **c** Phylogram of cileviruses and other related viruses for which sequence information is available. The tree was generated using a neighbor-joining algorithm using the RdRp domain and bootstrapped with 1000 replications. Branch support is presented as a percentage, and the bar represents the branch distance for the indicated number of substitutions. BNRBV, blueberry necrotic ring blotch virus (YP_004901701); CiLV-C, citrus leprosis virus C (YP_654568); CiLV-C2 (AGE82887); HGSV-2, hibiscus greenspot virus 2 (YP_004928118); LigRSV, Ligustrum ringspot virus (ADM47770); TMV, tobacco mosaic virus (ABN79257; used as outgroup). **d** Alignment of 5'- and 3'-terminal sequences of the cilevirus infecting hibiscus (Hib) and CiLV-C2 (Cit) in **a**. Nucleotide positions are in parentheses

virus described in this study represents the first documented case of a cilevirus established in the United States. A similar disease of hibiscus associated with an agent having cilevirus-like properties has previously been described in Brazil [5]. This agent has not been molecularly characterized and may be similar to the virus infecting hibiscus in Hawaii. Although it is unclear if the virus described in this study is also capable of infecting citrus and producing leprosis-like symptoms, the global trade in ornamental hibiscus may serve as a pathway for its international movement.

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