Identification and Characterization of *Citrus yellow vein clearing virus*, A Putative New Member of the Genus *Mandarivirus*

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ABSTRACT

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Molecular features and genomic organization were determined for *Citrus yellow vein clearing virus* (CYVCV), the putative viral causal agent of yellow vein clearing disease of lemon trees, reported in Pakistan, India, and more recently in Turkey and China. CYVCV isolate Y1 from Adana, Turkey, was used for deep sequencing analysis of the virus-induced small RNA fractions and for mechanical and graft inoculation of herbaceous and citrus indicator plants. A polyclonal antiserum was developed from CYVCV-Y1 purified from *Phaseolus vulgaris* and used in western blot assays to characterize the coat protein of CYVCV-Y1 and determine its serological relationship with related viruses. Contigs assembled from the Illumina sequenced short reads were used to construct

the whole genome of *Citrus yellow vein clearing virus* (CYVCV), consisting in a positive-sense RNA of 7,529 nucleotides and containing six predicted open reading frames. The CYVCV genome organization and size resembled that of flexiviruses, and search for sequence homologies revealed that *Indian citrus ringspot virus* (ICRSV) (*Mandarivirus*, *Alphaflexiviridae*) is the most closely related virus. However, CYVCV had an overall nucleotide sequence identity of $\approx 74\%$ with ICRSV. Although the two viruses were similar with regard to genome organization, viral particles, and herbaceous host range, CYVCV caused different symptoms in citrus and was serologically distinct from ICRSV. Primer pairs were designed and used to detect the virus by conventional and quantitative reverse transcription-polymerase chain reaction on yellow vein clearing symptomatic field trees as well as graft- and mechanically inoculated host plants. Collectively, these data suggest that CYVCV is the causal agent of yellow vein clearing disease and represents a new species in the genus *Mandarivirus*.

Yellow vein clearing disease (YVCD) was first observed in Pakistan in 1988 in lemon and sour orange trees (5,11). Symptoms in affected lemons included strong yellow vein clearing, leaf distortion, and occasionally, ringspots and veinal necrosis. The same symptoms were reported on lemon (*Citrus limon* L. Burm) 'Femminello Siracusano' and sour orange seedlings following graft inoculations with field-infected sources of YVCD (11). In 1997, YVCD-like symptoms were observed on citron Etrog, Rangspur lime, sour orange, and different lemon varieties in several citrus-growing areas of India. Graft inoculations to Etrog citron from affected field trees resulted in the reproduction of the symptoms (2). Recent reports indicate rapid spread of YVCD in Pakistan (15) and in Turkey (11,29,30), where the disease was observed for the first time in 2000 in lemon and sour orange trees (26,27). In 2009, lemon trees with YVCD symptoms were observed in Yunnan (China) (40).

Electron microscope examination of leaf dips from affected trees from Pakistan, India, and Turkey revealed the presence of flexuous filamentous virus-like particles, 13 to 14 nm in diameter with a modal length of 685 nm (2,11,27). Isolates of this virus, provisionally named *Citrus yellow vein clearing virus* (CYVCV),

were transmitted by mechanical inoculation to *Chenopodium quinoa* and *C. amaranticolor* and resulted in local lesions, while *Phaseolus vulgaris* and *Vigna unguiculata* reacted with systemic chlorosis, severe mosaic, blotching, and necrosis (2,30). Analysis of double-stranded RNA (dsRNA) profile of infected lemon and beans revealed the presence of the replicative forms of a putative viral genomic RNA and several potential subgenomic RNAs ranging from 0.8 to 10 kb in size (29,30). *Aphis craccivora* Koch and *A. spiraeicola* Patch transmitted the virus at high rates to beans after an acquisition and inoculation period of 24 h, whereas transmission from lemon to lemon plants remains to be ascertained (29,30). Although these biological properties of CYVCV have been reported, there is no information on the nucleotide sequence, organization, or structure of the viral genome.

Recently, next generation sequencing has emerged as a powerful approach for characterizing small interfering RNAs (siRNAs) generated by posttranscriptional silencing pathways. Upon virus infection, virus-specific siRNAs are produced by dicer-like (DCL) endoribonucleases for antiviral defense (7). *De novo* assembly of the short reads derived from the high-throughput sequencing of siRNAs allows reconstruction of the genome of uncharacterized or unknown viruses or of viruses that are too divergent to be detected by assays based on known sequences (3,9,18).

Deep sequencing analysis of siRNA fractions from a lemon plant infected with CYVCV strain Y1 from Turkey (30) enabled the assembly of the whole viral genome. Our findings indicated that the genome of CYVCV was sufficiently distinct from that of

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ICRSV (*Mandarivirus*, *Alphaflexiviridae*), a closely related virus, to warrant classification of CYVCV as a separate putative species in the genus *Mandarivirus*.

MATERIALS AND METHODS

Plant material. CYVCV-Y1 was obtained from a naturally infected lemon cultivar Kütiken showing typical yellow vein clearing symptoms from a commercial grove near Adana, Turkey, and was graft-propagated and maintained in a lemon seedling at Çukurova University (Adana, Turkey).

In 2011, CYVCV-Y1-infected budwood was used to graft inoculate citrus indicators Duncan grapefruit, Madam vinous, Mexican lime, Eureka lemon, and Parson's Special mandarin. The same indicators were mock-inoculated with virus-free blind buds as controls. Symptoms were monitored throughout the year. Mechanical transmission of CYVCV-Y1 to herbaceous hosts was done by homogenizing young symptomatic leaves of Eureka lemon in 50 mM Na-phosphate buffer (32) and rubbing the extract on celite-dusted leaves of *P. vulgaris* 'Dermason'.

Virus purification, antiserum production, and western blot. The virus was purified basically as described by Van Oosten (36). Symptomatic bean leaves (100 g) were homogenized with 400 ml of 0.1 M Na-citrate buffer, pH 8.3, containing 20 mM DIECA and 2 mM EDTA, and the slurry was filtered through gauze and centrifuged at 10,000 rpm for 10 min at 4°C. Triton X-100 (2%, vol/vol) was added to the supernatant and centrifuged for 1 h at 36,000 rpm. The pellet was resuspended in RB buffer, pH 8.3 (0.01 M Na-citrate, 0.5 M urea, and 0.1 M 2-mercaptoethanol), and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was layered on a 20% (wt/vol) sucrose cushion in 0.01 M Na-citrate, pH 8.3, and centrifuged at 27,000 rpm at 4°C for 2.5 h. The pellet was resuspended in RB buffer for 48 h at 4°C and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was layered on a 25 to 40% (wt/vol) Cs₂SO₄ gradient and centrifuged at 35,000 rpm for 3.5 h. A single opalescent band was observed in the gradient tube and was collected, dialyzed, and observed in the electron microscope. Virus yield in purified preparation was estimated spectrophotometrically as reported by Rustici et al. (32).

Purified virus (1 mg) was emulsified with an equal volume of Freund's incomplete adjuvant, and injected subcutaneously into a New Zealand white rabbit. Two booster intramuscular injections were made at 1-week intervals. Antiserum was collected through four bleedings beginning 21 days after the first injection and at 1-week intervals. The serum (CYVCV-As) was used directly or after cross-absorption with healthy plant extracts (1:2, vol/vol).

Western blot assays were performed using total protein extracts from CYVCV-Y1-infected Eureka lemon and beans, and symptomatic leaves from a mandarin infected by an isolate of ICRSV kindly provided and previously characterized by Dr. Baranwal (Indian Agricultural Research Institute, New Delhi).

Negative controls consisted of extracts from healthy plants. Assays were performed according to Hammond (12), i.e., 100 mg of leaf tissue was ground in 4 vol extraction buffer (0.5 M Tris-HCl pH 8.8, 2% SDS, 4% 2-mercaptoethanol, and 40% sucrose) and heat denatured at 100°C. Samples and a prestained protein molecular weight marker (New England BioLabs, Ipswich, MA) were loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel and electrophoresed. The finished gel was electroblotted on a polyvinyl difluoride membrane (PVDF, Immobilon-P, Millipore, Billerica, MA) following standard procedures. The membrane was soaked in blocking solution (TBS, 0.02 M Tris and 0.15 M sodium chloride; 5% non-fat dry milk; 1% bovine serum albumin; and 0.1% Tween-20) overnight at 4°C, and incubated in blocking buffer containing CYVCV-As at 1:200 dilution for 2 h at 37°C. After three washes in TBS with 0.1% Tween-20, the membrane was incubated for 1 h at room temperature with a 1:2,000 dilution of anti-rabbit immunoglobulin G (IgG)-AP conjugate in blocking

buffer (Sigma, St. Louis, MO). After washing, the membrane was incubated in BCIP-NBT liquid substrate system (Sigma) and the signal was visualized.

Electron microscopy. Partially purified virus preparation and crude extracts from leaf tissues of healthy and CYVCV-Y1-infected plants (beans and Eureka lemon) homogenized in 0.1 M phosphate buffer, pH 7.2, were negatively stained in 2% uranyl acetate and observed with a Philips Morgagni electron microscope. Immunoelectron microscopy tests were done according to Milne (21) using 1:50 and 1:100 dilutions of CYVCV-As.

High throughput sequencing of the small RNA fraction. A cDNA library was constructed using the siRNA fraction purified from 1 g of symptomatic leaves (20) collected from a Eureka lemon graft inoculated with CYVCV-Y1 and sequenced (reads of 36 cycles single-end) at the Institute of Applied Genomic, University of Udine (Italy) on the HiSeq2000 platform (Illumina, San Diego). The resultant sequence data sets were processed for adaptor trimming, and then ribosomal and transfer RNA sequences were removed by UEA siRNA toolkit (23). The filtered 18 to 26 siRNA sequences were characterized according to their size and polarity, and assembled *de novo* into larger contigs using Velvet Software 0.7.31 (37) with a k-mer of 17. The contigs were screened for sequence homologies using BLASTN (cutoff value 10⁻⁶) against the GenBank database (<http://www.ncbi.nlm.nih.gov/>).

The abundance of virus- and viroid-derived siRNAs was estimated by aligning the filtered reads on viroid genomes (HM042747, AF540966) and on the newly reconstructed viral genome (GenBank JX040635) using SOAPAligner.

Genome sequencing and phylogenetic analyses. CYVCV-related contigs were identified by homology to the genetically related ICRSV genome (AF406744). Based on the sequences of mapping contigs, eight primer pairs (Table 1) were designed to amplify cDNA fragments spanning the partially reconstructed

TABLE 1. List of the primers used in the study^a

Primer		PCR product size (bp)
Genome sequencing ^b		
7081fw	ACCTCACGATGGACCACGTT	479
7560rev	CAGAAAATGGAAACTGAAAGCCTG	
4914fw	CATTGCCTATCAACTTAAAGACGAGCT	1,313
6227rev	CAGAGTTGGTGCCGGTCTGAAC	
4323fw	GATCAACTGCGAGACCAACCCA	1,087
5410rev	GAATCGATGGGAAGACGCGCAGATGT	
3377fw	GGTTGGCCAACACTTGTCCCAT	1,120
4497rev	GGGATGGAGTGGTATTTGGCCTT	
2515fw	GCTACCTCGCTGAAGGTCTCGGTA	1,022
3537rev	GGTGTACATCACCTTGTCAGAGCATA	
1338fw	GCTCCCCCTCCCAGACATCTCAGA	1,314
2652rev	GTAATGCGTGATGGGCCATATGAGA	
668fw	GCCGCTACCCAGAGATATACCAATT	916
1584rev	GCGCTTGTITTTCTGAAGCTTGGTGA	
1fw	GAAAAGCAAACAKWAHCAWACACCCC	921
921rev	GGGCAAGAGCACTTGGGTATCT	
Detection ^c		
1fw	GAAAAGCAAACAGTAACAAACACACCC	921
921rev	GGGCAAGAGCATTGGGTATCT	
391fw	CGAATCCGCTGCGAGCTA	59
449rev	GGGAGTGCTTGGACAGGAGAT	

^a Primers are identified by the position of the first nucleotide at the 5'.

^b Primers to amplify cDNA fragments spanning the partially reconstructed putative *Citrus yellow vein clearing virus* (CYVCV) RNA genome.

^c Primers targeting the 5' region to detect CYVCV.

putative CYVCV RNA genome. The 5' and 3' termini were determined using the 5'/3' RACE kit (Roche, Basel, Switzerland) on the dsRNA template using the specific primers 7081fw/921rev and an oligo(dT) primer. Each cDNA fragment was cloned with Strataclone PCR cloning kit (Agilent Technologies, Santa Clara, CA) and a minimum of four recombinant clones sequenced.

The assembled full-length genome and each predicted ORF were subjected to phylogenetic analysis based on multiple or pairwise sequence alignments (MEGA5 package), and searched for conserved protein domains by using the NCBI conserved domain database (www.ncbi.nlm.nih.gov/cdd).

Development of PCR-based assays. Total RNA was extracted using a CTAB-based procedure (24) from 150 mg of leaves from CYVCV-Y1-affected Eureka lemon and from the control plants resuspended in 100 µl of nuclease-free water. The concentration of purified total RNA was adjusted to ca. 100 ng/µl and each sample was subjected to RT followed by conventional PCR and/or quantitative reverse transcription-PCR (qRT-PCR) assays. For cDNA synthesis, 3 µl of total RNA extract, 1 µl of random primers (0.5 µg/µl) (Roche, Basel, Switzerland) and 8.15 µl of RNase-free water were denatured for 5 min at 95°C and chilled on ice. M-MLV-RT buffer 1× (Invitrogen Life Technologies, Paisley, UK), 1.25 µl of 2.5 mM dNTPs, 1 µl of RNasin (40 U/µl), 1.2 µl of DTT (0.1 M), and 0.4 µl of M-MLV-RT (200 U/µl) were added and the samples were incubated for 1 h at 42°C. Conventional PCR was performed in a 25 µl volume of a reaction mixture containing 2.5 µl of cDNA, 1× GoTaq DNA Polymerase buffer (Promega, Madison, WI), 0.5 mM each dNTPs, 0.5 µM of each primer (1fw/921rev, Table 1) and 1 U of GoTaq DNA Polymerase (5 U/µl) (Promega). The amplification program was as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min, with a final elongation step of 7 min at 72°C. The PCR products were separated by electrophoresis in 1.2% agarose gel and DNA bands were visualized by staining with GelRed (Biotium, Hayward, CA). The resultant PCR product was purified, cloned with Strataclone PCR cloning kit, and sequenced.

One step qRT-PCR assays were performed by adding 1 µl of the total RNAs to the reaction mix containing 1× PerfeCTa SYBR Green SuperMix (Quanta Biosciences, Gaithersburg, MD), 0.4 µl of AMV (10 U/µl) (Promega) and 0.32 µM of each primer (391fw/449rev, Table 1) in a final volume of 12.5 µl. The cycling

profile consisted of a first two-step incubation at 46°C for 15 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 20 s, and 54°C for 40 s. Melt curve analysis was performed to determine the specificity of the amplicons produced.

The efficiency and sensitivity of the qRT-PCR assay were determined using 10-fold (from 1 to 10⁻⁵) serial dilutions of total RNA extract from CYVCV-Y1 in a total RNA extract of healthy Eureka lemon. Standard curves were generated using two replicates of each dilution in independent assays. To assess accuracy and reproducibility of results in the same run or in different runs, the quantification cycles (C_q) values from different standard dilutions were compared, and intra- and interassay coefficient of variation were calculated.

Validation of the specificity and sensitivity of the RT-PCR assay included seven samples from the citrus collection at Çukurova University (three symptomless and four symptomatic YCVD plants), healthy Eureka lemon seedlings, and positive and nontemplate controls (NTC). Additional controls included an isolate of ICRSV kindly provided by Dr. Baranwal as well as citrus tissues infected with *Citrus psorosis virus* (CPsV, *Ophiovirus*) isolate Ps101; *Citrus variegation virus* (CVV, *Ilarvirus*) strain cvv300; Citrus chlorotic dwarf-associated virus (CCDaV), isolate TK4; *Citrus leaf blotch virus* (CLBV, *Citricivirus*) isolate K1; and *Citrus tristeza virus* (CTV, *Closterovirus*), isolate IT-08 from the in planta citrus pathogen collection of the University of Bari.

RESULTS

Host range and symptoms. Mechanical inoculation of crude sap from Y1-infected Eureka lemon leaves resulted in severe systemic mosaic and leaf necrosis on *P. vulgaris*. Severe distortion, strong yellow blotchy chlorosis, and crinkling were observed on young leaves of Eureka lemon and sour orange; whereas, no symptoms were observed on Mexican lime, Duncan grapefruit, and Parson's Special Mandarin even though infection was confirmed by RT-PCR. Transient yellowing symptoms were observed on the young leaves of Madam vinous indicators which disappeared as the leaves aged. Filamentous particles were observed in leaf dips from all symptomatic herbaceous and citrus indicator hosts (Fig. 1A and B).

Fig. 1. Stained particles of *Citrus yellow vein clearing virus* (CYVCV) isolate Y1. CYVCV-Y1 negative stained particles from **A**, bean and **B**, lemon crude extracts. **C**, Decoration by CYVCV serum of Y1 from Eureka lemon crude extract. **D**, Flexuous filamentous particles shown in a partially purified preparation. Bars = 100 nm.

Virus purification, antiserum production, and western blot analysis. The virus sedimented in a single band in cesium sulfate gradients. Purified preparations had a maximum absorption at 260 nm with an $A_{260/280}$ ratio of 1.16 (corrected for light scattering).

Yield was estimated at 1.75 mg of virus per 100 g of leaf tissues. The antiserum, diluted 1:50 in phosphate buffer, clearly decorated virus particles trapped from crude extracts of CYVCV-Y1-infected bean and Eureka lemon leaves (Fig. 1C) and from a partially viral purified preparation (Fig. 1D). Measurements of virus particles gave a modal length of 650 nm with an evident “zipper-like” structure. Antiserum samples from the four bleedings were assayed separately in western blot. The strongest reaction was obtained with the fourth bleed, which detected a protein with a molecular mass of approximately 33 to 34 kDa from CYVCV-infected samples. No bands were observed in healthy or ICRSV-infected samples (Fig. 2).

High throughput sequencing and analysis of small RNA population. The library of siRNAs from the CYVCV-Y1-infected

plant contained a total of 3,220,571 raw reads. Adaptors removal, size selection (18 to 26 nts), and filtering of transfer and ribosomal RNAs trimmed the data set to 2,771,245 short reads. These siRNAs were subjected to *de novo* assembly and generated 450 contigs (Table 2) which ranged from 42 to 580 nts in length. BLASTN search identified one contig homologous to *Citrus viroid III* (CVdIII), two contigs homologous to *Citrus bark cracking viroid* (CBCVd), and 30 contigs that mapped on the ICRSV genome (AF406744) covering a genome sequence of 4,165 nts. No sequences homologous to other known viruses were found.

Short reads were then aligned with SOAPAligner on the above viral and viroidal genomes. A ratio of 56% positive to 44% negative polarities was observed; viral siRNAs 21 nts (40%) and 22 nts (27%) in size were predominate. These results are in agreement with previous reports on the hierarchical action of Dicer-like (DCL) 4 and DCL2 in the production of virus-derived siRNA of 21 to 22 nts in *Arabidopsis thaliana* (6).

Characterization of the CYVCV genome and phylogenetic relationships. Sequencing analysis of the PCR products (479 to 1,314 nts), obtained using eight selected primer pairs from the CYVCV-Y1-infected Eureka lemon, and the 5'- and 3'-RACE PCR products allowed assemblage of the entire genome sequence of CYVCV-Y1. It consisted of 7,529 nts, excluding the 3'-poly(A) tail (Fig. 3). No PCR products were amplified from healthy controls.

Computational analysis of the RNA genome sequence predicted six open reading frames (ORFs) (Fig. 3) on the positive strand, identifying a 5' untranslated region (UTR) of 78 nts and a 3'UTR of 36 nts. No ORFs were predicted on the negative strand. The number, length, and position of the ORFs determined for the CYVCV-Y1 genome resembled that of ICRSV isolate K1 (AF406774) (33). An overall nucleotide identity of 74% was estimated by pairwise alignment of the two viral genomes.

All six ORFs of CYVCV-Y1 start with an ATG codon and terminate either with a TAA stop codon (ORFs 1, 4, and 5) or with a TGA stop codon (ORFs 2, 3, and 6). Nucleotide and amino acid sequence analysis of ORF1 (nt 79 to 5028, 1,649 amino acid [aa]) showed 72 and 81% identity with ICRSV ORF1 at the nucleotide and amino acid level, respectively. CYVCV-Y1 ORF1 encodes a putative RNA-dependent RNA-polymerase (RdRp) and contains all replication (REP)-associated conserved domains (Fig. 3). Specifically, the viral methyltransferase domain (pfam01660) was found in the N-terminal region (aa 39 to 329), including the conserved motifs I-IV of “Sindbis-like” viruses (31). The domain pfam13532 of AlkB-like proteins members of the 2-oxoglutarate- and Fe(II)-dependent oxygenase superfamily (3) was identified at amino acid position 463 to 662. The viral RNA helicase 1 domain (pfam01443), in the central part (aa 926 to 1157), included the NTP binding helicase motif GAGGSGKS (aa 930 to 937) and the other five motifs conserved in nucleic acid helicases (34). The conserved domain of RdRp_2 (pfam00978) (17) at aa 1214 to 1649, included the highly conserved GDD motif located at aa 1520 to 1522.

Fig. 2. Western blot analysis with 4th Y1-antiserum bleed (1:200) against total proteins extracted from *Citrus yellow vein clearing virus* (CYVCV) isolate Y1-infected beans (lane 2) and Eureka lemon (lane 3), from healthy beans (lane 4) and eureka lemon (lane 5), and from symptomatic leaves of a mandarin infected by ICRSV (lane 6); Lane 1, prestained protein molecular weight marker (New England BioLabs, Ipswich, MA).

TABLE 2. Virus- and viroid-related 18-26 small interfering RNA and contigs obtained from the *Citrus yellow vein clearing virus* (CYVCV) isolate Y1 library

Origin	Reads ^a	% ^b	Contigs
CYVCV	138,195	5.00	47
<i>Citrus viroid III</i>	4,027	0.14	1
<i>Citrus bark cracking viroid</i>	4,581	0.17	2
Other ^c	2,624,442	—	417
Total	2,771,245 ^d	—	450

^a Redundant reads obtained by SOAPAligner.
^b Percentage over the total reads.
^c Reads not matching with viral and viroidal sequence.
^d Total reads after trimming of the adaptors and filtering for ribosomal and transfer RNAs.

Fig. 3. Schematic representation of the RNA genome of *Citrus yellow vein clearing virus* (CYVCV) isolate Y1. Open reading frames and conserved domains identified using conserved domain database search at NCBI are reported. REP, replication-associated polyproteins; M, viral methyltransferase domain; A, AlkB-like domain; H, viral helicase domain; R, RNA-dependent RNA polymerase 2 domain; TGB, triple gene block; CP, capsid protein; and 23K, encoded putative protein of 23 kDa.

ORF1 is followed by a triple gene block (TGB), i.e., ORF2 (nt 5035 to 5712), ORF3 (nt 5690 to 6016), and ORF4 (nt 5943 to 6125), involved in cell-to-cell movement (25) of fovea-, carla-, and potexviruses (16,39). ORF2 encodes a 25 kDa peptide which contains a large viral helicase 1 domain (pfam01443) including the NTP-binding helicase motif GAGKT (aa 30 to 34) (22), and the motif DEY (aa 76 to 78) of the catalytic site of ATPase (4). ORF2-encoded polypeptide showed 69% identity both at the nucleotide and amino acid sequence level, with the ORF2 product of ICRSV (AF406744). ORF3 and ORF4 encoding a 12 kDa (108 aa) and a 6.4 kDa (60 aa) putative protein, respectively, had 68% aa identity with the TGBp2 and TGBp3 homologous proteins of ICRSV. Indeed, the conserved domains, associated with plant viral movement proteins of potexviruses, hordeviruses, and carlaviruses (pfam01307) and the '7kDa viral coat protein' of carlaviruses and potexviruses (pfam029495) were found within the translation product of ORF3 (3 to 103 aa) and ORF4 (2 to 59 aa), respectively.

Based on the predicted amino acid sequence and, in agreement with western blotting results, ORF5 at nt 6148 to 7125 codes for the 34 kDa (325 aa) coat protein (CP), identified by the conserved domain (pfam00286: aa 140 to 278) of potexvirus and carlavirus CPs, including the RX----FDF (8), VWN (aa 226 to 228), PPANV (aa 236 to 240) and AAFDF (aa 252 to 256) motifs conserved amongst carla-, fovea-, allexi-, and potexviruses. Identity levels between the putative CP encoded by CYVCV-Y1 and the CP gene of the ICRSV isolates available in the NCBI database indicated a shared 97 to 98% amino acid identity with the CP gene of ICRSV-Ah (AY255007) and ICRSV-Pu (AY255008), and 80% amino acid identity with the CP gene of ICRSV-K1, ICRSV-DI (AA072983), and ICRSV-Ab (AA072988). Based on their CP sequence, isolates ICRSV-Ah and ICRSV-Pu were described by Hoa and Ahlawat (14) as highly divergent Indian strains of ICRSV. A phylogenetic tree constructed with these CP sequences placed the isolates into two distinct groups (Fig. 4), with ICRSV-Ah and ICRSV-Pu clustering with the CP gene of CYVCV-Y1.

ORF6 (6825 to 7493 nt) overlaps ORF5 and encodes a hypothetical 23 kDa peptide (aa 222) containing the conserved domain pfam05515 (aa 68 to 222) of the nucleic acid-binding proteins of positive-strand ssRNA viruses, including the zinc finger-like sequence (aa 175 to 190) conserved amongst members of the genera *Carlavirus* and *Allexivirus* (10,13,35). The nucleotide and amino acid identity with the protein encoded by ORF6 of ICRSV ranged from 76 to 70%, respectively.

Phylogenetic trees from deduced amino acid sequences of ORF1 (RdRp) and ORF5 (CP) compared with that from repre-

sentative members of the families *Alphaflexiviridae* and *Betaflexiviridae*, positioned CYVCV-Y1 on the same branch as ICRSV, but in a clade clearly distinct from members of the other genera (Fig. 5).

The complete CYVCV-Y1 genome sequence has been deposited in GenBank under the accession number JX040635.

Detection of CYVCV. Using the newly acquired sequence data, both conventional and qRT-PCR assays were developed for CYVCV detection. The virus was readily identified in all symptomatic plants, including the four symptomatic sources from Turkey, all graft-inoculated indicator plants. A single band of the expected size of 921 nts was observed after PCR amplification with primers 1fw/921rev, but not from healthy Eureka lemons or the NTC (Fig. 6). Likewise, there was no amplification from citrus plants infected with ICRSV, CPsV, CVV, CCDaV, CBLV, or CTV. Cloning and sequencing confirmed that the 921-nt fragment amplified by PCR shared 100% nucleotide sequence identity with the newly assembled RNA molecule (data not shown). The one step qRT-PCR assay with primer pair 391fw/449rev produced Cq between 14 and 20 from YVCD-affected field samples and from graft-inoculated indicator plants; while no fluorescence (Cq = 0; no melt peak) was obtained from healthy controls, symptomless field samples, CPsV-, CVV-, CCDaV-, CBLV-, CTV-, or ICRSV-infected plants, or NTC. A single melt peak with a Tm of 78.50°C representing the melting temperature of the specific CYVCV amplicon was observed in all samples that had a Cq value higher than 0. Standard curves generated using a serial 10-fold dilution of a total RNA extract purified from CYVCV-Y1 exhibited an efficiency [E = 10(-1/slope) - 1] of 97.5%, corresponding to a slope of -3.384. The overall coefficient of variation for all standard dilutions was less than 5%. The results (Cq, E, slope) indicated that the developed assay was specific, sensitive, and reproducible.

DISCUSSION

YVCD, a virus-like disorder of lemon, was first reported in Pakistan in 1988 (5,11) and was associated with the presence of a filamentous virus in symptomatic plants. However, investigations of the nature and aetiology of this disease did not begin until the early 2000s in India and Turkey (2,26,27,29,30). Examination of diseased tissues by electron microscopy consistently revealed the presence of filamentous virus particles. The virus, tentatively named CYVCV in 2003 by Alshani et al. (2), was successfully transmitted to several herbaceous host plants, from which it was purified and used for the production of a polyclonal antiserum.

Fig. 4. Phylogenetic tree generated by the neighbor-joining method from the alignment of the deduced amino acid sequence of the capsid protein genes of *Citrus yellow vein clearing virus* (CYVCV) and isolates of *Indian citrus ringspot virus* (ICRSV), ICRSV-K1 (NP203547), ICRSV-DI (AA072983), ICRSV-Ah (AA072985), ICRSV-Pu (AA072987), and ICRSV-Ab (AA072988). Bootstrap values for 1,000 replicates are indicated at the main branches. Branch length is proportional to number of amino acid changes.

Western blot assays detected a 32 kDa protein, assumed to be the major structural protein of CYVCV. The polyclonal antiserum against CYVCV-Y1 produced in this study showed that CYVCV is serologically distinct from ICRSV, in agreement with the enzyme-linked immunosorbent assay and immunosorbent electron microscope assays reported by Alshami et al. (2).

The advent of next generation sequencing (NGS) technologies has greatly advanced our ability to comprehensively investigate diseases of unknown aetiology and expedited the entire process of virus discovery, identification, viral genome sequencing, and subsequently, the development of routine assays for new viral pathogens (19). In this work, the whole genome of CYVCV was obtained by assembling contigs recovered by sequencing the siRNAs associated to an YVCD-affected source and by sequencing the amplicons generated by conventional PCR. The analysis of the

NGS data provided a wide range of sequences in search of systemic pathogens present in the sample analyzed. siRNAs generated by two viroids (CVdIII and CBCVd), unwittingly present in the CYVCV-Y1 source, were subsequently detected and confirmed by RT-PCR assays (data not shown). Both viroids were previously reported in Turkey (28) and were associated to naturally dwarfed Meyer lemon grafted on sour orange. There are no reports or observations that these viroids are involved in YVCD syndrome.

Comparative sequence analysis of the newly assembled CYVCV genome allowed determination of its phylogenetic relationships and taxonomic affiliation. Specifically, analysis of the full CYVCV sequence showed that the genome organization is typical of flexiviruses, with six ORFs, with homologies with the RdRp (ORF1), TGB (ORFs 2 to 4), CP (ORF5), and P23 (ORF6) of

Fig. 5. Phylogenetic trees generated by the neighbor-joining method from the alignment of the **A**, entire RNA-dependent RNA-polymerase or **B**, coat proteins of *Citrus yellow vein clearing virus* (CYVCV) and members belonging to the families Alpha- and Betaflexiviridae, using MEGA (Version 5). Bootstrap values for 1,000 replicates are indicated at the main branches. Branch length is proportional to number of amino acid changes. Accession numbers of reference sequences are as follows: *Apple chlorotic leaf spot virus* (ACLSV, NC_001409); *Apple stem grooving virus* (ASGV, NC001749); *Blackberry virus E* (BVE, JN053266); *Blueberry scorch virus* (BBSv, NC 003499); *Botrytis virus X* (BOTVX, NC005132); *Indian citrus ringspot virus* (ICRSV, NC003093); *Lolium latent virus* (LoLV, NC010434); *Potato virus X* (PVX, NC011620); *Sclerotinia sclerotiorum debilitation-associated RNA virus* (SsDaRV, NC_007415); *Shallot virus X* (NC003795); *Sweet potato chlorotic fleck virus* (SPCFV, NC_006550); and *White ash mosaic virus* (WAMV, DQ412998).

Fig. 6. Agarose gel electrophoresis of reverse transcription-polymerase chain reaction products of *Citrus yellow vein clearing virus* (CYVCV) infected field trees and indicator plants. Lane 1, 1-kb DNA ladder (Fermentas, Burlington, Ontario, Canada); lanes 2 to 5, field symptomatic trees; lanes 6 to 11, graft-inoculated Eureka lemon, sour orange, Madam vinous, Duncan grapefruit, Mexican lime, and Parson's special mandarin; lanes 12 to 14, field symptomless trees; lanes 15 to 16, healthy Eureka lemon; and lane 17, nontemplate control.

ICRSV. The six predicted ORFs contained all the specific conserved domains typical of proteins encoded by flexiviruses. Molecular criteria for species demarcation within the genera of the family *Alphaflexiviridae* (1) indicate that distinct species have less than ca. 72% nucleotide or less than 80% amino acid identity between their entire CP or RdRp genes. In this case, CYVCV-Y1 CP showed 75 and 80% identity, at the nucleotide and amino acid level, respectively, with the CP of ICRSV, the most closely related virus; whereas ORF1, which encodes the putative RdRp, shared 72 and 81% identity at the nucleotide and amino acid level, respectively, with the comparable protein of ICRSV. Phylogenetic trees, constructed on the multiple alignment of these two ORFs with representative members of the families *Alphaflexiviridae* and *Betaflexiviridae*, consistently positioned CYVCV and ICRSV on the same branch, clearly apart from all other members of the families *Alphaflexiviridae* and *Betaflexiviridae*.

However, the two viruses have different natural host plants and cause two distinct diseases: ICRSV is associated with a disease of kinnow mandarin consisting of chlorotic rings on leaves, premature leaf drop, branch dieback, and decline; whereas CYVCV is characterized by vein clearing, leaf crinkling and yellowing, poor growth, and reduced fruit production. Moreover, CYVCV symptoms on citrus indicator plants were different from those reported for ICRSV.

Additional search for sequence similarity indicated that the CP gene of the highly divergent ICRSV-Ah and ICRSV-Pu isolates (14) had strong phylogenetic relationship with that of CYVCV-Y1, providing support that they may be Indian isolates of CYVCV rather than ICRSV.

The findings that the virus-derived siRNAs detected in the Y1 source were solely related to CYVCV and that the virus, using the newly developed RT-PCR based assays, was detected in all natural and graft-inoculated symptomatic lemons strongly supports previous evidence that CYVCV is the putative causal agent of the homonymous disease.

The genus *Mandarivirus* currently includes only ICRSV, and thus, no specific criteria for species demarcation have been defined (1). The sequence identities (nucleotide and amino acid) for the CP and RdRp genes between CYVCV and ICRSV fulfill the range accepted for the differentiation of a new species. In conclusion, the serological, biological, and molecular data presented support the hypothesis that CYVCV is a distinct new species. Thus, this virus is proposed as a new species in the genus *Mandarivirus*.

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