Virology 432 (2012) 162–172

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Identification of a single-stranded DNA virus associated with citrus chlorotic dwarf disease, a new member in the family *Geminiviridae*

Giuliana Loconsole [a], Pasquale Saldarelli [b], Harshavardhan Doddapaneni [c], Vito Savino [a,b], Giovanni P. Martelli [a,b], Maria Saponari [b,*]

[a] Dipartimento di Biologia e Chimica Agroforestale ed Ambientale, Università di Bari "Aldo Moro", Via Amendola 165/A, 70126 Bari, Italy
[b] Istituto di Virologia Vegetale del CNR, UOS Bari, Via Amendola 165/A, 70126, Bari, Italy
[c] Carver Center for Genomics, Department of Biology, The University of Iowa, Iowa City, IA 52242, USA

ARTICLE INFO

Article history:
Received 7 May 2012
Returned to author for revisions
28 May 2012
Accepted 8 June 2012
Available online 1 July 2012

Keywords:
Citrus chlorotic dwarf disease
High-throughput sequencing
Geminivirus
ssDNA

ABSTRACT

In the attempt to identify the causal agent of Citrus chlorotic dwarf disease (CCDD), a virus-like disorder of citrus, the small RNA fraction and total DNA from symptomatic citrus plants were subjected to high-throughput sequencing. DNA fragments deriving from an apparently new geminivirus-like agent were found and assembled by NGS to re-construct the entire viral genome. The newly identified virus has a circular single-stranded DNA genome comprising five open reading frames (ORFs) with sequence homologies with those encoded by geminiviruses. PCR and qPCR assays were successfully used for determining its presence in the CCDD-affected plants obtained by graft propagation. The larger genome size (3.64 *vs.* 2.5–3.0 kb) and a number of differences in its structural organization, identified this virus as a highly divergent member of the family *Geminiviridae*, to which the provisional name of Citrus chlorotic dwarf-associated virus (CCDaV) is assigned.

Introduction

Citrus, one of the economically most important fruit crops in the world, is susceptible to several arthropod-transmitted pathogens and graft-transmissible agents that cause a number of different diseases, some of which are still aetiologically uncharacterized (Bové, 1995). This is the case of citrus chlorotic dwarf disease (CCDD), a graft-transmissible disorder first observed in the mid-1980s in southern Mediterranean Turkey (mainly in the Mersin and Adana provinces) where about 85% of the country's total citrus production is concentrated (Çinar et al., 1993, 1994; Kersting et al., 1996; Korkmaz et al., 1994a). A survey conducted in 1994, revealed that CCDD affected 49% of the citrus trees in Mersin, 0.5% in Adana, and was not present in the Hatay province (Korkmaz et al., 1994a). Recent records, however, indicate that CCDD incidence has grown to 60–70% in Mersin, 1–2% in Adana, and to about 5% in Hatay (European Food Safety Authority, 2008).

CCDD is considered to be the most serious disease of citrus in the Turkish areas of occurrence (Çinar et al., 1993, 1994; Korkmaz et al., 1994a), where severe losses on different species (50% in grapefruit) are reported, due to the reduction in the number and size of the fruits. So far, only sweet orange varieties show some tolerance to the disease. Infected symptomless plants may act as source of inoculum (European Food Safety Authority, 2008). CCDD is particularly detrimental to *Citrus limon* (L.) Burm. (lemon), *C. paradisi* Macf. (grapefruit), *C. aurantium* (L.) (sour orange), *C. macrophylla* (Webster) (Alemow), *C. jambhiri* Lush (rough lemon), *C. deliciosa* Ten. (common mandarin), *C. unshiu* (Makino) Marc. (Satsuma mandarin), *C. clementina* Hort. Ex Tan. (clementine) and *C. reticulata* × *C. paradisi* (Tangelo). Disease symptoms include crinkling, curling, inverted cupping, deformation and distortion of the leaves, which may also show chlorotic patterns and variegation, and reduced size. Affected young trees display a bushy vegetation and stunted appearance, due to the shortened internodes.

The putative CCDD agent is transmitted by the bayberry whitefly *Parabemisia myricae* Kuwana (Homoptera: Aleyrodidae) in a persistent or semi-persistent manner (Kersting et al., 1996; Korkmaz et al., 1994a, 1994b, 1995; Korkmaz and Garnsey, 2000). Although CCDD is not reported from any citrus-growing country other than Turkey, its vector is present in the USA (California and Florida), Venezuela, Malaysia, China, Taiwan, Japan, Israel, Cyprus, Spain and Italy (Kuwana, 1928; Takahshi, 1952; Anonymous, 1978, 1986; Sternlicht, 1979; Longo et al.,1990; Rapisarda et al., 1990, 1991; Garrido, 1991; Garcia Segura et al., 1992). Thus, if the disease would be introduced in a citrus-growing area where the vector is present, it could spread and cause damage. This risk is high for Mediterranean countries close to Turkey where the vector is present, Cyprus first, then Israel, Italy and Spain.

CCDD is currently identified by graft- or slash-inoculation to sensitive citrus species, such as *C. macrophylla*, rough lemon or

* Corresponding author. Fax: +39 0805443608.
E-mail address: m.saponari@ba.ivv.cnr.it (M. Saponari).

0042-6822/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2012.06.005

sour orange (Korkmaz and Garnsey, 2000). Symptoms on the inoculated plants develop at 20–25°C but become stronger at temperature of 30–35 °C (Korkmaz et al., 1995). The agent of the disease is still unknown. Ultrastructural investigations of thin-sectioned citrus leaf tissues did not reveal the presence of identifiable virus particles (Korkmaz and Garnsey, 2000; Howd et al., 2002) and mechanical transmissions to herbaceous hosts yielded the recovery of the necrovirus Olive latent virus (OLV-1) whose involvement in the aetiology of the disease was excluded because of its occurrence in both symptomatic and symptomless plants. (Martelli et al., 1996).

Metagenomics, a molecular technique that involves shotgun sequencing of environmental samples, circumvents the biases of classic viral identification methods and has revolutionized the exploration of viral diversity (Delwart, 2007; Edwards and Rohwer, 2005). This procedure has been used for characterizing virus communities in the environment (Breitbart et al., 2002; Dinsdale et al., 2008; Lopez-Bueno et al., 2009), in individual plants (Muthukumar et al., 2009) and animals (Ng et al., 2009). The advantage of metagenomics is that it allows the identification of the whole viral community in a host, including viruses that are too divergent to be detected by nucleic acid-based techniques. Small RNA fractions, purified total nucleic acids (RNA, DNA, double-stranded RNA), or virus particles can be used as template for direct deep sequencing.

De novo assembly of small RNAs (sRNAs) or DNA fragments originating from a replicating viral genome allows compiling large continuous fragments (contigs). Hitherto unknown viruses have been identified through this approach, by assembling the short reads derived from the high-throughput sequencing of the sRNA population or double-stranded RNAs (dsRNAs) isolated from virus-infected plants (Kreuze et al., 2009; Al Rwahnih et al., 2009; Zhang et al., 2011; Giampetruzzi et al., 2012).

As reported in this paper, the use of deep-sequencing allowed the discovery of a novel DNA virus with a single-stranded circular genome 3640 nts in size (GenBank accession number JQ920490) and properties fitting those of members of the family *Geminiviridae*, for which the provisional name of Citrus chlorotic dwarf-associated virus (CCDaV) is proposed.

Results

High-throughput sequencing, analysis of libraries and de novo assembly of small RNA and DNA fragments

Library s1, representative of the sRNA population extracted from the CCDD-affected TK4 lemon and sequenced using Illumina technology, contained a total of 2.77 million raw short reads. After adapters removal, size selection (18–26 nt) and filtering for transfer and ribosomal RNAs, a dataset of 1.8 million of total short reads was obtained and subjected to *de novo* assembly, generating a total of 136 contigs, ranging from 33 to 367 nts. BLAST search of the resultant contigs in the NCBI virus database, using a cutoff e-value of 10^{-6} for BLASTN and 10^{-4} for BLASTX, identified 9 contigs with amino acid homologies to genes encoded by different members of the family *Geminiviridae*, and 10 contigs with nucleotide sequence homologies to four known citrus-infecting viroids (Table 1).

Library s2 generated a total of 40.9 million raw reads, with 81.8% of these reads with a value of Q30 or higher. Filtered reads were directly used for *de novo* assembly, which produced 244,427 contigs ranging from 60 to 3974 nts. BLASTX analysis of these contigs confirmed that TK4 lemon contained virus-like sequences deriving from a putative novel DNA virus. Specifically, three partially overlapping contigs 494, 1377 and 1775 nt in size were identified and aligned to obtain a DNA molecule of 3614 nts that showed amino acid homologies with different members of the family *Geminiviridae* (Table 1).

Discovery of a new viral DNA molecule, its genome organization and genetic relationships with members of the Geminiviridae family

Overlapping PCR products were obtained using five primer pairs (Table 2) designed according to the sequence of the newly assembled partial DNA molecule. The sequences from these PCR products, ranging from 329 to 2657 nt in size, were used to extend the entire genome to a sequence of 3640 nts which showed 99% identity with the sequence assembled by next generation sequencing (NGS) (data not shown). The amplification product yielded using primers 2559fw/1576rev suggested that the target DNA molecule had a circular structure. No products were amplified from any of the healthy plants, confirming the specificity of the primers selected.

The CLC genomic workbench software predicted the presence of five ORFs in the CCDaV DNA (Fig. 1), three of which were coded on the 5′ half of the virion-sense strand (ORF1, 2 and 3) and two on the 5′ half of the complementary-sense strand (ORF4 and 5). Sequence analysis identified in the viral genome the presence of the sequence TAATATTAC conserved in all geminiviruses and positioned within an intergenic region (IR) containing a short palindromic sequences capable of forming hairpin-like structures (Fig. 1).

Computer search for transcription regulatory sequences detected three putative promoters (P1–P3): P1, located in the IR downstream the hairpin-like structure at nt position 58–81, contains a TATA box (nt 76–81) and an upstream GC-rich region, which may act as activator sequence (Bensimhon et al., 1983); P2 (nt 1059–1099), positioned upstream of ORF3, contains a TATA box at nt 1094–1097 and an activator region starting at nt 1059; P3, detected in the IR upstream the hairpin-like structure (nt 3509–3485), has a TATA box at nt position 3490–3493 and an activator sequence starting at nt 3509.

The CCDaV genome has the following structural organization and expresses products whose nature was identified by BLASTP analysis (Table 3):

ORF1 (V2, nt 194–613), which initiates with an ATG codon and terminates with a TAA stop codon, encodes a potential 15.3 kDa peptide of 139aa containing the conserved domains of geminivirus V2 protein. At nt position 89, this protein has a conserved cysteine residue found to be critical for the suppression of silencing in *Tomato yellow leaf curl virus* (TYLCV) (Wartig et al., 1997). Interestingly, beginning at nt position 27, the aa sequence LKCVE representative of the motif LxCxE was found; this motif is conserved across geminivirus replication proteins and interacts with the retinoblastoma-related protein (RBR) to induce conditions permissive for viral DNA replication (Gutierrez, 2000).

ORF2 (V1, nt 417–1181), which initiates with an ATG codon and terminates with a TAA stop codon, partially overlaps ORF1 and encodes a 27.9 kDa protein of 254 aa identified as the putative coat protein (CP). Alignment of the conserved geminivirus CP domain with the V1 predicted product disclosed the presence of the potential nuclear export and localization signals (aa 95–245).

ORF3 (V3, nt 1211–2131) which, like ORF1 and ORF2 initiates with an ATG codon and terminates with a TAA stop codon, codes for a 33.6 kDa protein of 306aa containing the BC1 conserved domain of proteins involved in the cell-to-cell movement of genomic DNA of bipartite begomoviruses (Brown et al., 2011).

Two putative polyadenylation signals (AATAAA) for transcription termination (Birnstiel et al., 1985) occur in the 5′ half of the virion-sense strand comprising the three above ORFs. The first initiates at nt position 1180 and the second at nt position 2247.

Table 1
Virus and viroid contigs originated from two libraries identified by BLASTN and BLASTX analysis towards NCBI virus and viroid database.

Contig length (nt)	Virus/viroid sequences	e-value	Contig length (nt)	Virus sequences	e-value
Contigs from sRNA library			*Contigs from total DNA library*		
341	Hypothetical protein of South African cassava mosaic virus-[M12] (CAD195099)	2.91336E-25	494	BC1 of Pepper golden mosaic virus (AAY16456)	5.34017E-39
146	Rep protein of Chickpea chlorosis virus-B (ADR30017)	1.39493E-19	1775	AC1 gene product [Asystasia begomovirus 1] replication associated protein YP_004958233	8.5492E-54
119	Movement protein of Sida micrantha mosaic virus (CBH28934)	8.16125E-17	1377	Coat protein of Tomato yellow leaf curl Kanchanaburi virus (ABA00485)	2.87672E-36
367	BC1 of Pepper golden mosaic virus (AAY16456)	2.47873E-11			
302	Hypothetical protein of Barley dwarf virus (CAP57992)	3.30283E-11			
105	Replication-associated protein of Spinach curly top Arizona virus (YP_004207925)	6.16143E-07			
183	Coat protein of Cotton leaf curl Gezira virus-Cameroon (CAR65236)	9.74598E-07			
94	C2 of Spinach curly top Arizona virus (YP_004207924)	6.27105E-06			
71	Movement protein of Rhynchosia golden mosaic Havana virus (ADN84044)	5.43815E-05			
242	Citrus exocortis viroid isolate GBI2, complete genome (GQ260198)	4.9587E-124			
142	Hop stunt viroid isolate Mor24, complete genome, genomic RNA (FR865479)	9.59802E-70			
140	Citrus bent leaf viroid isolate IbI4, complete genome, (GQ260202)	1.16928E-68			
111	Hop stunt viroid isolate Mor61, complete genome, genomic RNA (FR865480)	6.47281E-53			
107	Citrus exocortis viroid isolate Hn-G1, complete genome (GU592444)	2.11597E-46			
96	Citrus dwarfing viroid isolate LS-4, complete genome (JF812069)	8.99734E-45			
106	Citrus dwarfing viroid isolate LS-4, complete genome (JF812069)	1.62676E-41			
50	Citrus dwarfing viroid isolate H10–7, complete genome (JF970268)	8.43426E-20			
66	Citrus bent leaf viroid clone Hb-C1–2, complete genome (JF742600)	6.48425E-15			
66	Citrus bent leaf viroid clone Hb-C1–2, complete genome (JF742600)	3.14593E-06			

ORF4 (C1, nt 3420–2611), the first of the two slightly overlapping ORFs located in the 5′ half of the complementary-sense strand, has ATG and TAG initiation and termination codons, respectively, and codes for a viral Replication protein (Rep) of 269aa with a molecular mass of 29.6 kDa. The alignment of the central and catalytic domains of geminivirus Replication protein with the C1-encoded peptide showed, in its amino-terminal half, the presence of three conserved sequence motifs (Nash et al., 2011) characteristic of rolling-circle initiators (Fig. 2): (i) motif I (FLTY) starting at aa position 16, required for specific dsDNA binding; (ii) motif II (HLH) at aa position 58, a metal-binding site that may be involved in protein conformation and DNA cleavage; (iii) motif III (YLMKD) at aa position 104, which is the catalytic site for DNA cleavage, that forms with the hydroxyl group of the Y residue a covalent bond with the 5′ phosphoryl group of the cleaved DNA strand. The recently described geminivirus Rep sequence (GRS)-conserved motif containing 12 consensus aa, which is required for the initiation of rolling-circle replication (Nash et al., 2011), was found between motif II and III (aa 75–97).

ORF5 (C2, 2707–2300nt), which starts with a GAG and ends with a TGA codon, encodes a putative 14.9 kDa protein of 136aa. BLASTP analysis of the deduced aa sequence of its predicted product showed 48% to 51% identity with the 3′ end of the C1:C2 Rep protein of mastreviruses (Table 3), and the presence of the Walker ATPase domain (Walker et al., 1982), consisting of two conserved separate NTP-binding motifs: (i) Walker A (GPSRSGKT), starting at aa 19 and (ii) Walker B (LYNVIDDI), starting at aa 52. These motifs are crucial components of the nucleotide-binding site. A single polyadenylation signal was identified on the negative strand at nt positions 2240–2366.

In phylogenetic trees constructed with the amino acid sequences of CP domain (Fig. 3A), Rep catalytic and central domains (Fig. 3B), CCDaV was consistently located between the clusters comprising members of the genera *Begomovirus* and *Mastrevirus* in a distinct branch close to the *Begomovirus* clade. This position was retained in the tree generated with sequences of the BC1 domain (Fig. 3C).

A single circular genomic DNA is associated with CCDaV

Electrophoresis of the rolling circle amplification (RCA) products from healthy and CCDaV-infected plants (Fig. 4A) revealed in both the presence of a single high molecular weight band that migrated at different rates (compare lanes 1 and 2 in Fig. 4A).

Table 2
Primers used in this study, identified by the position of the first nucleotide at the 5′.

		PCR product size (bp)
Genome walking		
1409 fw	GGGAATGTATTGGTTGTGGTACACGA	2550
341 rev	CAGGGCTCCTCCTACGGGATT	
221 fw	GATTTGCCCGAGAGTTTGTTCGGTT	1321
1542 rev	GGTCCTTCAACGAAAAATACGAACA	
1128 fw	GATTTGCCCGAGAGTTTGTTCGGTT	2094
3222 rev	AACGGGTCGAAACACAGAAC	
2559 fw	TGGTCGTTGTAGTCGTGGAA	2657
1576 rev	TTCACAACCCTATAGTATACACACCA	
Oligoprobes synthesis		
3202fw	GTTCTGTGTTTCGACCCGTT	444
6rev	GGGATTCGCATGGATAGCTCATCCAA	
417fw	ATGGTGAGTACCAGGAGTGGAGGA	203
622rev	ATGGGCCCTTACACCCCGGAGGAA	
1409fw	GGGAATGTATTGGTTGTGGTACACGA	383
1791 rev	CTGGGCCGGGTCGTTAAAACACTA	
3016fw	CACGTCCCTCCACAGTTTTT	404
3421rev	ATGGCTTCCACTTCCTCTAGCTT	
Amplification of the entire molecule		
1213fw	GGACGTCAAGATTTGGTGTACA	3640
1236rev	GTAACACCAAATCTTGACCGTCCA	
Detection		
3202fw	GTTCTGTGTTTCGACCCGTT	444
6rev	GGGATTCGCATGGATAGCTCATCCAA	
1583fw	GTGTTGAAGGGTTCTCATTTTTGTC	68
1650rev	CTACTTGATTTAGCCGCGCTTAG	

Single digestions with restriction enzymes *Xho*I, *Nco*I, *Sac*I and *Spe*I (Fig. 4A, lanes 3–6) of the RCA product obtained from the CCDaV-infected TK4, produced a common band nearly 4 kb in size, that had the same electrophoresis mobility of the PCR product corresponding to the entire viral DNA molecule (Fig. 4A, lane 7). However, in the SacI digestion, a second band *ca.* 1.2 kb in size was observed (Fig. 4A, lane 5) which was also found in the digested RCA product from a healthy plant (Fig. 4A, lane 8).

Southern blot assays using digoxigenin (Dig)-labeled probes that targeted sequences in the predicted ORFs V2, V1, V3 and C1 (Table 2), showed a clear-cut and strong hybridization signal corresponding to the nearly 4 kb band observed in the RCA products from TK4 digested with *Xho*I, *Nco*I, *Sac*I and *Spe*I, and the PCR product corresponding to the entire viral DNA (Fig. 4B–E, lanes 3–7). Moreover, a signal corresponding to a single high molecular weight DNA band was observed in the undigested RCA product from TK4 (Fig. 4B–E, lane 2) whereas no signals were detected in correspondence of the 1.2 kb band in TK4 nor in the digested and undigested RCA product from the healthy plant (Fig. 4B–E, lane 1).

Development of tools for CCDaV detection

Both conventional and quantitative real time PCR (qPCR) assays successfully detected CCDaV in all symptomatic plants, including the six sources from Turkey and all indicator plants graft-inoculated in our screenhouse (data not shown).

A single band of the expected size (444 nts) was observed after electrophoresis of conventional PCR products obtained with primers 3202fw/6rev. No bands were visualized in the mock graft-inoculated indicators, in the negative or non-template (NTC) controls. Cloning and sequencing confirmed that the 444 nts fragment shared 100% nucleotide sequence identity with the newly assembled circular DNA molecule (data not shown).

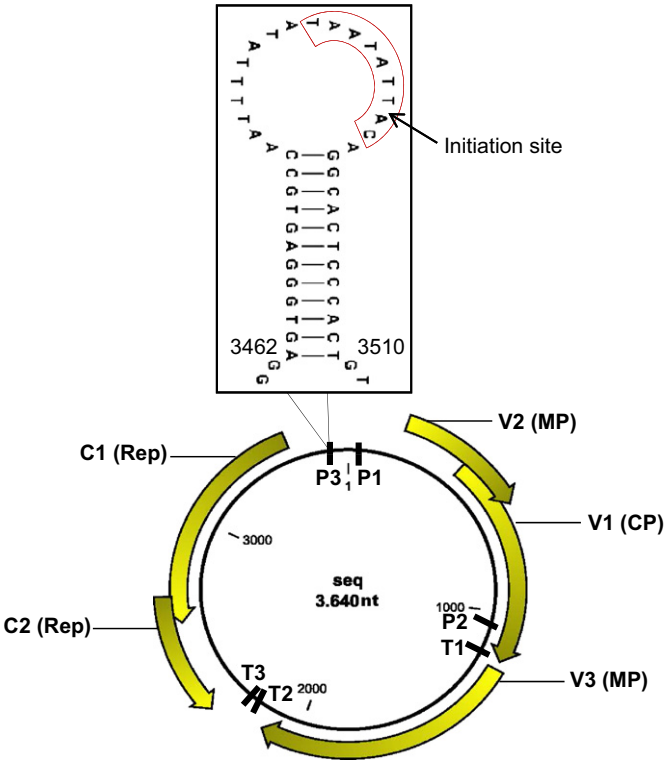

Fig. 1. Genomic organization of Citrus chlorotic dwarf-associated virus (CCDaV) showing the arrangement of five predicted open reading frames (ORF) with relative predicted proteins and the position of the TAATATTAC sequence (inside the stem-loop), the initation site for replication conserved across geminiviruses. ORF V2 (nt 194–613), ORF V1 (nt 417–1181) and ORF V3 (nt 1211–2131) are comprised in the virion-sense strand, ORF C1 (nt 3420–2611) and ORF C2 (nt 2707–2300) in the complementary-sense strand. Stem loop from nt 3462 to 3510. The positions of transcription (P1, P2, P3) and termination (T1, T2, T3) sites are indicated.

qPCR assay with primer pair 1583fw/1650rev produced quantification cycles (Cq) comprised between 12 and 18 for the symptomatic samples collected from the original graft-inoculated sources TK1 to TK6, while no fluorescence (Cq=0; no melt peak) was measured from healthy plants and NTC. A single melt peak with a Tm of 77 °C representing the melting temperature of the specific CCDaV amplicon was observed in all infected samples. Standard curves generated using a serial 10-fold dilutions of a total DNA extract purified from TK4, exhibited an efficiency ($E=10^{(-1/\text{slope})}-1$) of 90%, corresponding to a slope of −3.800 (Fig. 5). The overall coefficient of variation (CV) for all standard dilutions was less than 5%. The results (Cq, *E*, slope) indicated that the developed assay was specific, sensitive and reproducible.

Association of CCDaV with chlorotic dwarf symptoms

CCDD-affected sources from Turkey were free from *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV) and *Citrus variegation virus* (CVV), but were positive for *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* IIIb (CVd-III variant b), *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd) as shown by RT-PCR assays.

When indicator plants (citron Etrog, Madam vinous, Eureka lemon, sour orange, Mexican lime and Duncan grapefruit) were mechanically inoculated with an extract prepared by grinding in water CCDaV-infected leaf tissues from TK4, Etrog plants reacted with typical CEVd symptoms, (Fig. 6E) whereas no reactions were observed in any of the other indicators. By contrast, all slash-inoculated indicators tested positive for CBLVd, CVd-IIIb, HSVd and CEVd in standard RT-PCR assays, but none of them for CCDaV using

Table 3
Comparison of the amino acid sequences encoded by the five predicted ORFs of the CCDaV genome with the most closely related geninivirus sequences.

ORFs	Homologous protein (GenBank accession number, Genus)	Identity (%)	Conserved domain (NCBI CDD)
ORF1 (V2)	AV2		pfam01524 – Geminivirus_V2, associated with systemic infection
	Tomato yellow leaf curl virus (P27269, Begomovirus)	26	
	Mungbean yellow mosaic virus (Q9YPS6, *Begomovirus*)	25	
	Tomato yellow leaf curl Sardinia virus (P61511, *Begomovirus*)	29	
	African cassava mosaic virus (P14975, *Begomovirus*)	28	
ORF2 (V1)	CP		pfam00844 – Geminivirus coat protein/nuclear export factor
	Tomato golden mosaic virus-Yellow vein (P03560, *Begomovirus*)	33	
	Bean golden yellow mosaic virus (P0CK33, *Begomovirus*)	33	
	Squash leaf curl virus (P27444, *Begomovirus*)	32	
	Tomato yellow leaf curl Sardinia virus (P27257, *Begomovirus*)	31	
ORF3 (V3)	BC1		pfam00845 – Geminivirus BC1 movement protein.
	Tomato mottle virus (Q06660, *Begomovirus*)	48	
	Tomato golden mosaic virus-Yellow vein (P03566, *Begomovirus*)	48	
	Abutilon mosaic virus (P21946, *Begomovirus*)	44	
	Potato yellow mosaic virus (P27267, *Begomovirus*)	47	
ORF4 (C1)	Rep C1 of Tomato leaf curl virus (P36279, *Begomovirus*)	38	pfam00799 – Geminivirus Rep catalytic domain. pfam08283 – Geminivirus Rep central domain.
	Rep AC1 of African cassava mosaic virus (P14982, *Begomovirus*)	36	
	Rep AC1 of Abutilon mosaic virus (P21947, *Begomovirus*)	37	
	RepA of Tobacco yellow dwarf virus (P31617, *Mastrevirus*)	31	
ORF5 (C2)	Rep C1:C2		Walker ATPase domain
	Been Yellow Dwarf Virus (O395522, *Mastrevirus*)	51	
	Tobacco yellow dwarf virus (P31618, Mastrevirus)	50	
	Wheat Dwarf Virus (Q67622, Mastrevirus)	49	
	Miscanthus streak virus (Q67590), Mastrevirus)	46	

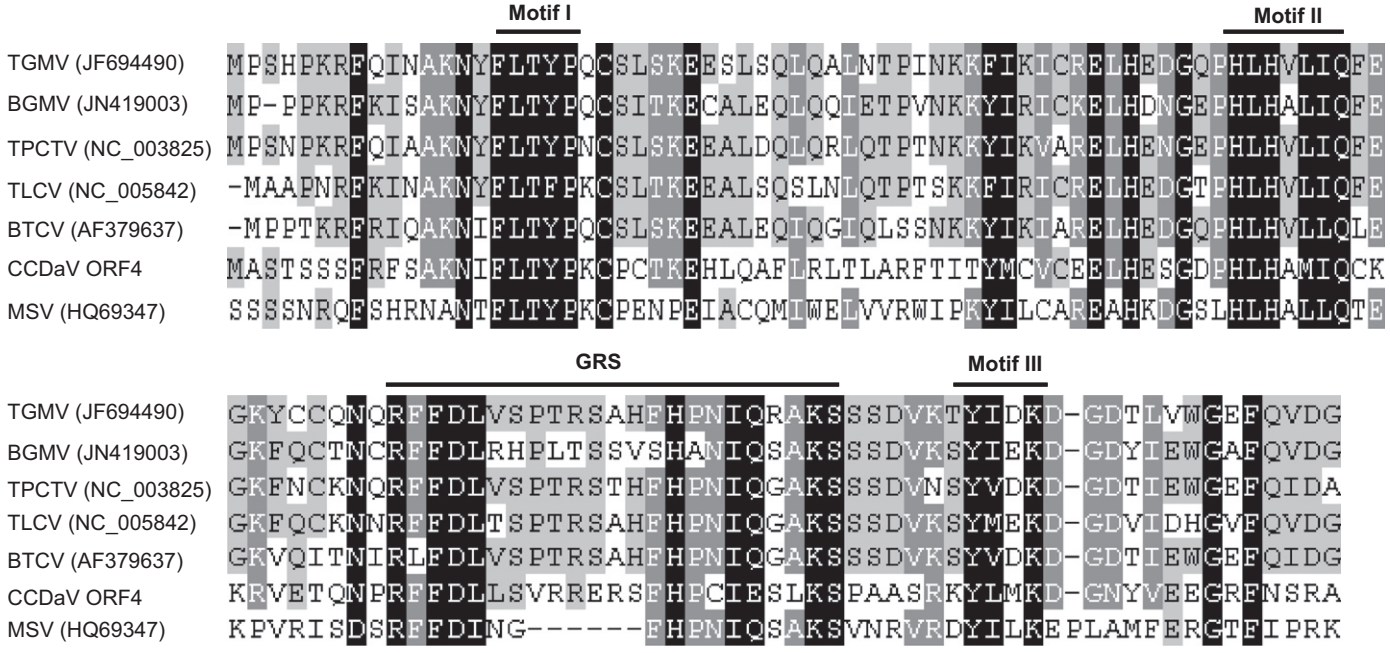

Fig. 2. MEGA 5 amino acid sequence alignment showing motifs I, II, III and GRS in the N-terminus of the putative protein translated from CCDaV ORF C1 and of Rep proteins of members of the genera *Begomovirus* [*Tomato leaf curl virus* (TLCV), *Bean golden mosaic virus* (BGMV), *Tomato golden mosaic virus* (TGMV)], *Curtovirus* [*Beet curly top virus* (BCTV)], *Topocovirus* [*Tomato pseudo-curly top virus* (TPCTV)] and *Mastrevirus* [Maize streak virus (MSV)].

the newly developed qPCR assay. All sour orange, Mexican lime, Eureka lemon and Duncan grapefruit seedlings graft-inoculated with bark pieces from TK4 reproduced the typical CCDD symptoms (Fig. 6A–D) and tested positive for the complex of viroids as well as for CCDaV. Graft-inoculated seedlings of Madam vinous were symptomless but positive for CCDaV and all viroids.

Discussion

The nature (DNA), type (circular) and organization of the CCDaV genome, and the presence of geminivirus conserved domains strongly support the notion that this virus is a member of the family *Geminiviridae*. Currently, this family comprises four genera, *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*, differing in genome organization, host range and transmission modalities (Fauquet and Stanley, 2003; Brown et al., 2011). The genus *Mastrevirus* includes highly divergent and distinctive leafhopper-transmitted species infecting both monocotyledonous and dicotyledonous plants. It has a monopartite genome 2.6–2.8 kb in size and four ORFs. Curtoviruses and topocuviruses infect only dicotyledonous plants to which they are transmitted by treehoppers. Both possess a monopartite genome with 6–7 ORFs and a size of 2.9–3.0 kb (curtoviruses) or 6 ORFs and a size of 2.8 kb (topocuviruses). Members of the genus *Begomovirus* are

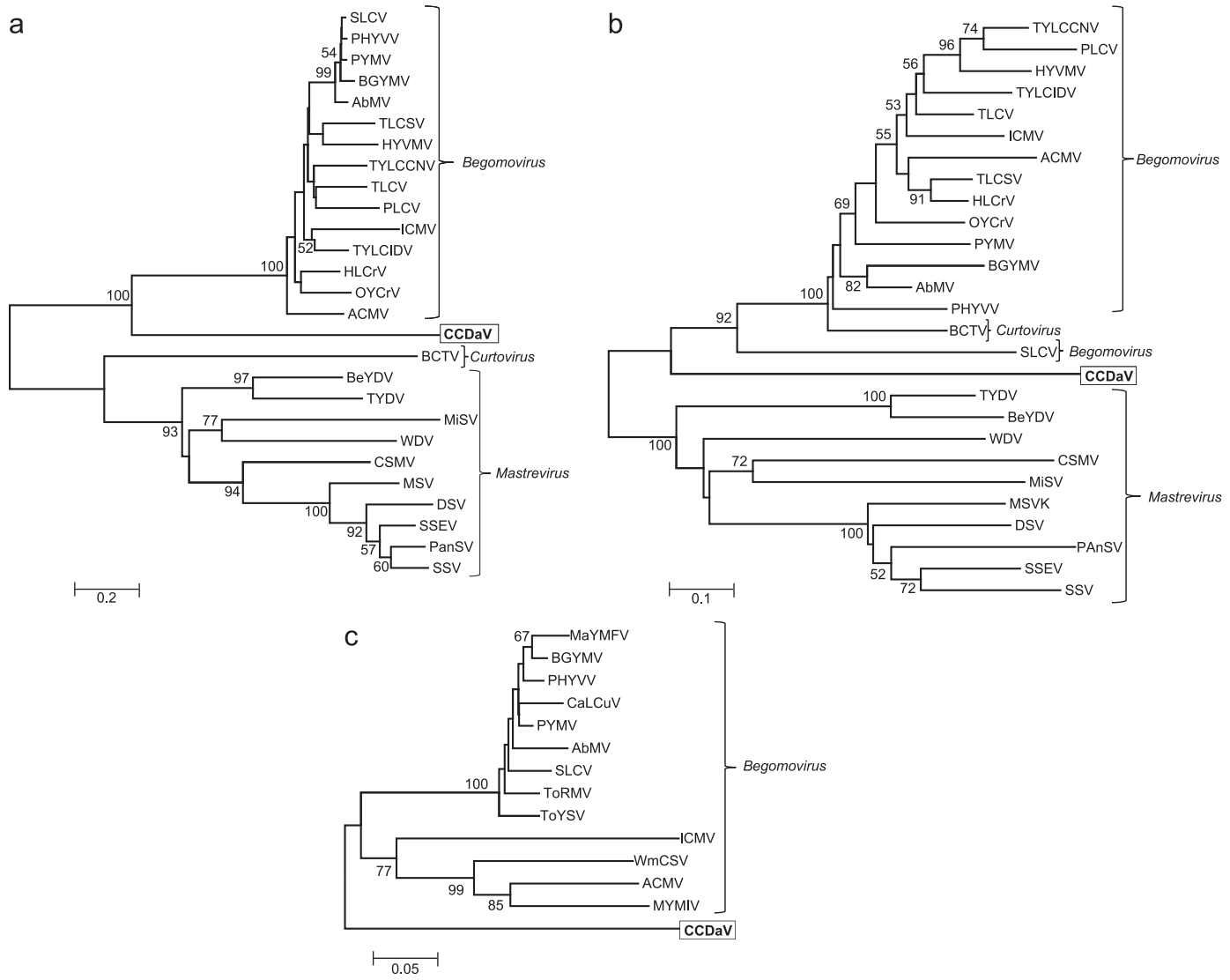

Fig. 3. Phylogenetic trees generated by the Neighbor-Joining method from the alignment of the amino acid sequence of the conserved coat protein domain (A), Rep catalytic and central domains (B) and BC1 domain (C) of geminiviruses using MEGA (Version 5). Bootstrap values for 1000 replicates are indicated at the main branches. Branch length is proportional to number of amino acid changes. Accession numbers of reference protein sequences are: (3A-3B): *Abutilon mosaic virus* (AbMV, 48429218, 172046797); *African cassava mosaic virus* (ACMV, 81935134, 137408); *Bean golden yellow mosaic virus* (BGYMV, 378948297, 137407); *Bean yellow dwarf virus* (BeYDV, 81921095, 91980535); *Beet curly top virus* (BCTV, 210681, 50897840); *Chloris striate mosaic virus* (CSMV, 116739, 137410); *Digitaria streak virus* (DSV, 81937170, 9626691); *Hollyhock leaf crumple virus* (HLCrV, 81956319, 18091747); *Honeysuckle yellow vein mosaic virus* (HYVMV, 81943357, 20153405); *Indian cassava mosaic virus* (ICMV, 301343698, 60418955); *Maize streak virus* (MSV, 116760, 137411); *Miscanthus streak virus* (MiSV, 116757, 222132); *Okra yellow crinkle virus* (OYCrV, 152143576, 152143577); *Panicum streak virus* (PanSV, 81993086, 137414); *Pepper huasteco yellow vein virus* (PHYVV, 9626082, 549195); *Pepper leaf curl virus* (PLCV, 81975402, 6031139); *Potato yellow mosaic virus* (PYMV, 222460, 137415); *Squash leaf curl virus* (SLCV, 9630693, 137416); *Sugarcane streak Egypt virus* (SSEV, 81922390, 2809395); *Sugarcane streak virus* (SSV, 30027718, 75570065); *Tobacco yellow dwarf virus* (TYDV, 399273, 401432); *Tomato leaf curl virus* (TLCV, 21040374, 549197); *Tomato leaf curl Sudan virus* (TLCSV, 81949780, 22073998); *Tomato yellow leaf curl China virus* (TYLCCNV, 123812367, 11037046); *Tomato yellow leaf curl Indonesia virus* (TYLCIDV, 123880258, 110628254); *Wheat Dwarf Virus* (WDV, 75569937, 137420). (3C): *Abutilon mosaic virus* (AbMV, 1657748); *African cassava mosaic virus* (ACMV, 72292281); *Bean golden yellow mosaic virus* (BGYMV, 5823270); *Cabbage leal curl virus* (CaLCuV, 123846513); *Indian cassava mosaic virus* (ICMV, 123841203); *Macroptilium yellow mosaic Florida virus* (MaYMFV, 81949782); *Mungbean yellow mosaic India virus* (MYMIV, 81960080); *Pepper huasteco yellow vein virus* (PHYVV, 268308992); *Potato yellow mosaic virus* (PYMV, 339727845); *Squash leaf curl virus* (SLCV, 123839879); *Tomato yellow spot virus* (ToYSV, 123833593); *Tomato rugose mosaic virus* (ToRMV, 81969218); *Watermelon chlorotic stunt virus* (WmCSV, 81976247).

transmitted by the whitefly *Bemisia tabaci* to dicotyledonous hosts and have either a monopartite (6 ORFs) or a bipartite genome (8 ORFs) of 2.5–2.6 kb and ~5.2 kb in size, respectively.

The arrangement of the five predicted ORFs within the CCDaV genome resembles only in part to those reported for other geminiviruses (Bisaro, 1996; Yazdi et al., 2008; Varsani et al., 2009; Briddon et al., 2010; Brown et al., 2011). For instance, the localization of ORF V3, whose predicted product has the properties of a putative movement protein, clearly distinguishes CCDaV from other members of the family, a third ORF in the virion-sense strand is only found in the curtovirus genome, but in a different position.

The overlapping ORFs V2 and V1 are positioned like those of begomoviruses, topocuviruses and mastreviruses denoted similarly V2 and V1. Although a low identity at the amino acid level (25–33%) was found with the proteins encoded by the comparable genes of begomoviruses, the conserved domains of the V2-MP and CP/nuclear export factor proteins were found in CCDaV ORFs V2 and V1, respectively (Table 3), which justified their naming.

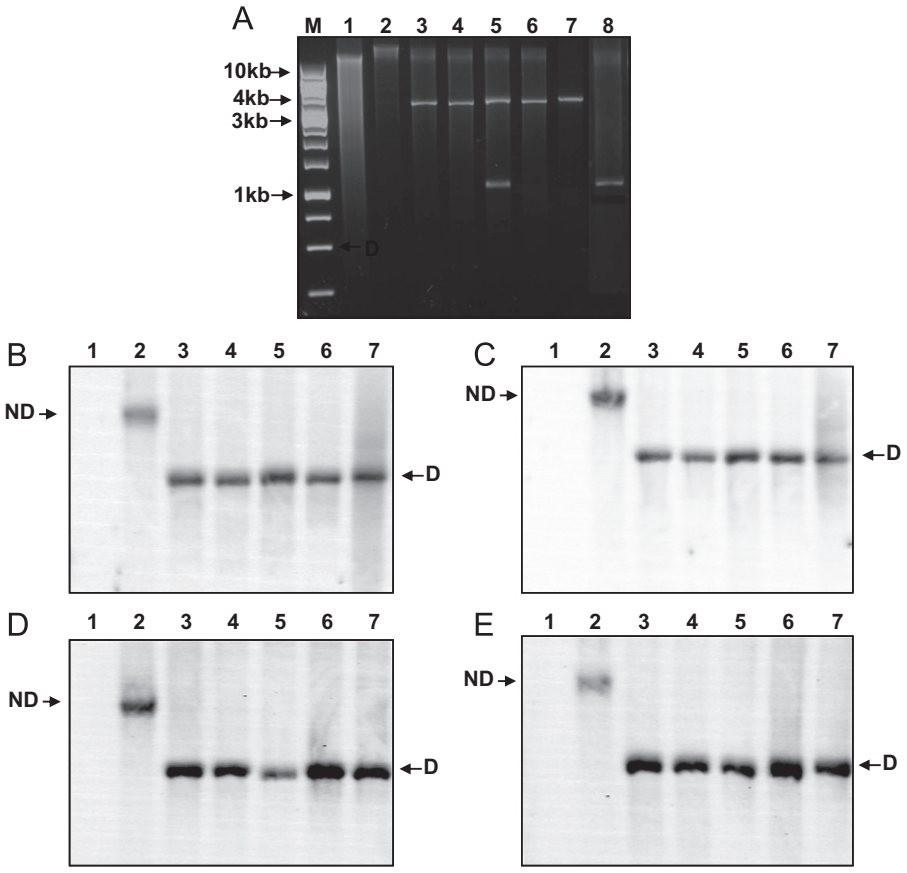

Fig. 4. Agarose gel and southern blot hybridizations. A. Agarose gel electrophoresis (1%) of RCA products digested (D) or not digested (ND) with restriction enzymes. Lanes 1–2, ND RCA product from healthy and from CCDaV-infected TK4 lemon, respectively; lanes 3–6, RCA products from CCDaV-infected TK4 digested with *Xho*I, *Nco*I, *Sac*I and *Spe*I, respectively; lane 7, PCR control corresponding to the entire genomic molecule cloned in the pSCA vector; lane 8, RCA product from healthy plant digested with *Sac*I from. M: 1kb DNA ladder (Fermentas, Burlington, Canada). B to E. Southern blot hybridization with probes specific to target sequences within ORFs V2 (B), V1 (C), V3 (D) and C1 (4).

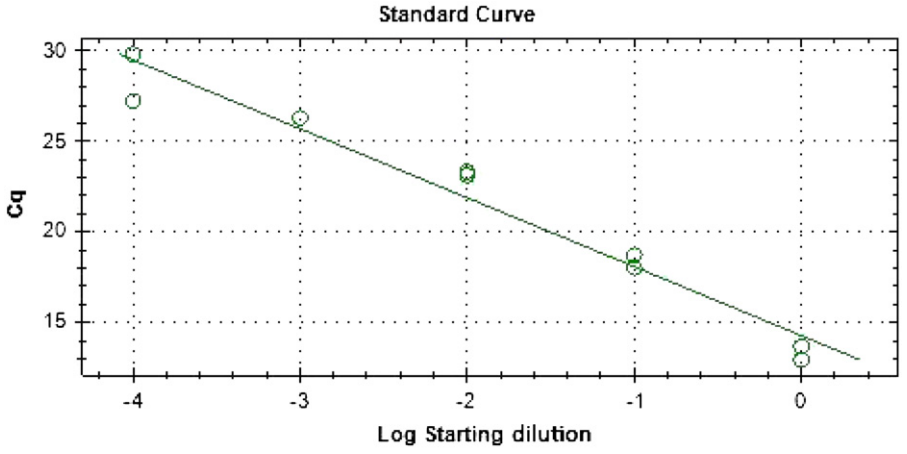

Fig. 5. Standard curve, linear regression (R2) and slope obtained in qPCR with primers for CCDaV detection, using 10-fold serial dilution (from 1 to 10^{-4}) of total DNA extract from TK4 lemon.

The two complementary-sense and partially overlapping ORFs C1 and C2 occur in the same position as the C1 and C2 genes of mastreviruses. As to C1, despite the low amino acid sequence identity (31–38%) of the predicted product of this ORF with the Rep C1/AC1 of begomoviruses and Rep A of mastreviruses, it contained the geminivirus-conserved catalytic and central domains of a Replication protein (Table 3), as well as the Rep motifs I, II, III and GRS. The position of ORF C2 matches that of the C2 gene of begomoviruses and curtoviruses, and its amino acid sequence shows identities ranging between 46% and 51% with the C-terminus of Rep C1:C2 of mastreviruses. Similarly to several C2 genes of monopartite geminiviruses, this ORF does not have an ATG initiation codon (Marja et al., 1994) and contains the conserved Walker A and B motifs (NTP binding motif).

Regarding the potential expression strategies of the CCDaV predicted genes, the identification and the localization of the three putative promoters and polyadenylation signals suggest that P1 could be likely responsible for the transcription of a

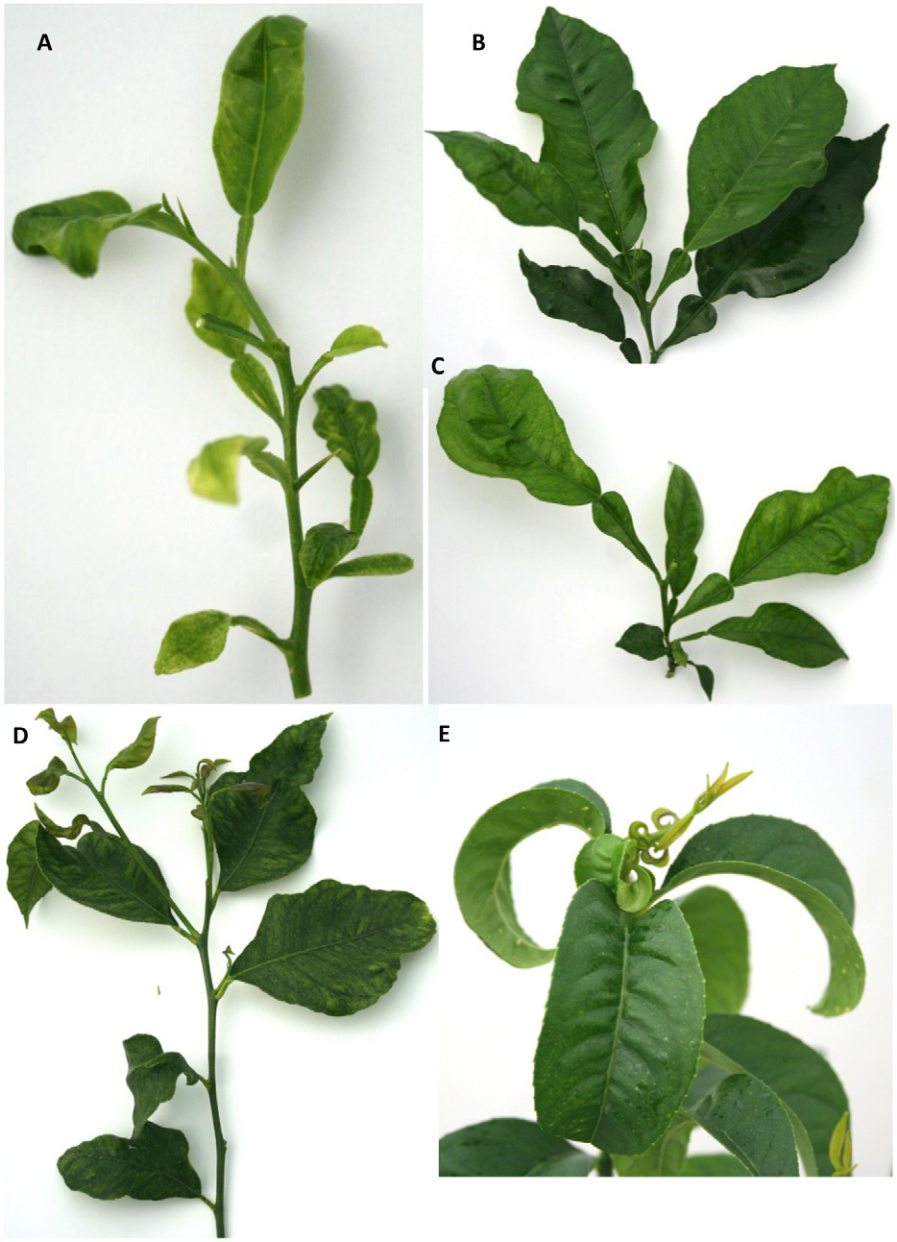

Fig. 6. Symptoms on the leaves of the indicator plants inoculated using the Citrus chlorotic dwarf- affected TK4 lemon. Crinkling, curling, inverted cupping, deformation and chlorosis shown by graft-inoculated Mexican lime (A), sour orange (B), Duncan grapefruit (C) and Eureka lemon (D). Slash-inoculated citron Etrog (E) showed the typical leaf epinasty induced by *Citrus exocortis viroid*.

polycistronic RNA from V2 and V1, while the first polyadenylation signal on the virion-sense strand could represent the transcription termination for this RNA. Similarly, the promoter P2 and the second polyadenylation signal on the virion-sense strand could be responsible for the transcription and termination of the V3 transcript. On the complementary sense strand, the promoter P3 and the single polyadenylation signal present, could represent the regulatory sequences responsible for the transcription and termination of C1 and C2 in a putative polycistronic transcript.

Rolling circle amplification and southern blot assays using four different Dig-labeled oligoprobes, proved that a single circular DNA molecule was associated with CCDaV. This finding is supported by the fact that as with other monopartite geminiviruses, this single DNA molecule, seems to contain all viral information necessary for replication (C1 and C2), encapsidation (V1), cell-to-cell and systemic spread (V2 and V3).

Besides the low genome sequence homologies, three relevant molecular and two biological features differentiate CCDaV from members of the family *Geminiviridae*: (i) genome size, which is larger (3.6 kb) than those of other geminiviruses (2.5–3.0 kb); (ii) the presence in the virion-sense strand of ORF V3 coding for a movement protein, which is only found in the curtovirus genome, where it encodes a protein with a different function and is located in a different position; (iii) the existence in V2 (MP-like protein) of the conserved motif LxCxE, associated to the Rep-encoding genes of geminiviruses; (iv) the nature of the putative vector, *i.e.* a whitefly (*Parabemisia myricae*) that thrives on woody plants and differs taxonomically from *B. tabaci*, the vector of begomoviruses; (v) the nature of the host (*Citrus* spp.) which, contrary to the plants (primarily herbaceous, more rarely woody shrubs) that are commonly infected by geminiviruses, is a perennial woody species.

Even if further experiments need to be done to determine the role of CCDaV in the aetiology of CCDD, the assays carried out showed a strict correlation amongst the presence of this virus in plants showing a typical CCDD symptomatology, whereas the complex of viroids isolated from infected plant did not induce any of the CCDD symptoms. The lack of symptoms on Madam vinous indicator plants infected with CCDaV, confirmed the tolerance to CCDD previously reported for the sweet orange varieties.

In conclusion, although the molecular features strongly support the notion that CCDaV is a geminivirus of good standing, the differences in genome size and structure, and the distant phylogenetic relationships with other members of the family *Geminiviridae*, does not allow its allocation in any of the existing genera of this family.

Materials and methods

Plants material and transmission trials

Cuttings from six distinct CCDD-affected citrus trees were collected in the course of a survey conducted in Turkey in late 1990s and grafted onto sour orange and Eureka lemon seedlings (TK1 to TK6) grown in an insect-proof screenhouse at the University of Bari. All graft-inoculated plants showed typical CCDD symptoms within a few weeks. These plants were screened by RT-PCR for the presence of *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV) and *Citrus variegation virus* (CVV) (Saponari et al., 2008; Loconsole et al., 2009, 2010), and six known citrus viroids (Ito et al., 2002). Among the six CCDD sources, accession TK4 (lemon) was selected for biological assays, molecular tests and deep sequencing analysis. Three seedlings each of citron Etrog, sour orange, Mexican lime, Madam vinous, Eureka lemon and Duncan grapefruit were slash-inoculated as described by Thomas et al. (2010). At the same time three replicates of the same indicator plants were grafted-inoculated with bark pieces. Furthermore, a set of citrus indicator plants were mock graft-inoculated with virus-free blind buds as healthy controls. Symptoms on the foliage were recorded three and six months post-inoculation, and the presence of viroids assessed (Ito et al., 2002) three months after inoculation.

Small RNA and DNA enrichment and sequencing of cDNA libraries

A cDNA library (s1) was constructed using the small RNA fraction purified from 1 g of symptomatic leaves following the protocol described by Lu et al. (2007). The recovered cDNA was sequenced (reads of 36 cycles single-end) at the Institute of Applied Genomic, University of Udine (Italy), on the HiSeq2000 platform (Illumina, San Diego, USA).

Genomic DNA (50 ng) was used in a fragmentation reaction with the Nextera DNA Sample Prep Kit (Epicentre Biotechnologies, Madison, USA) to prepare an Illumina sequencing-compatible library. The resultant library s2 was sequenced as 51 nt single reads on a single lane of the Illumina Genome Analyzer IIx at The University of Iowa.

Sequence analysis of the libraries

Adaptors, ribosomal and transfer RNA sequences were removed from the s1 sequence dataset by using UEA siRNA toolkit (Moxon et al., 2008). The filtered small RNA sequences, 18 and 26 nt in size, were then assembled *de novo* into larger contigs using Velvet Software 0.7.31 (Zerbino and Birney, 2008) with a k-mer of 17. Similarly, the short reads from the s2 library were assembled using a k-mer of 23. The s1 and s2 resultant contigs were screened for sequence homologies using BLASTN (cutoff value 10^{-6}), BLASTX (cutoff value 10^{-4}) and BLASTP against the GenBank Virus Reference Sequence database (http://www.ncbi.nlm.nih.gov/).

Genomic DNA was reconstructed by manually aligning homologous contigs on the most closely related sequences found in the GenBank database. Then, the sequence gaps were filled by sequencing the DNA fragments amplified with primers designed for genome walking. Amplicons were cloned with Strataclone PCR cloning kit (Agilent Technologies, Santa Clara, USA) and a minimum of four clones per fragment sequenced.

The reconstructed genome was subjected to standard sequence analyses: (i) prediction of the open reading frames (ORFs) and assessment of the presence of transcriptional control signals, using the CLC software (CLCbio, Aarhus, Denmark); (ii) evaluation of phylogenetic relationships based on multiple sequence alignments (MEGA5 package); (iii) search for conserved protein domains and sequence motifs prediction using the NCBI conserved domain database (CDD) (http://www.ncbi.nlm.nih.gov/cdd).

Rolling circle amplification (RCA) and southern blotting

A CTAB-based extraction procedure (Murray and Thompson, 1980) was used to recover total DNA from 150 mg of leaves from the CCDD-affected TK4 and from healthy lemon plants. The replicative form of the viral genome (RF-DNA) was enriched as described by Palmer et al. (1998). RCA (Inoue-Nagata et al., 2004) was performed using an Illustra^{TM} TempliPhi 100 Amplification Kit (GE Healthcare, Uppsala, Sweden) according to manufacturer's instructions. The circular DNA-enriched preparations were used as template to amplify the entire reconstructed genome and for Southern blot hybridization, after enzymatic digestions. Specifically, 4 μl of RCA product from TK4, diluted 1 to 40, were subjected to PCR using the Takara Taq^{TM} (Takara Bio, Shiga, Japan) and 0.25 μM of each primer (1213fw/1236rev). Amplicons of the expected size were purified, cloned with Strataclone PCR cloning kit (Agilent Technologies, Santa Clara, USA) and sequenced. One recombinant clone was then chosen and used as template to generate the control PCR amplicon used in the Southern blotting experiments. For Southern blot hybridization, 10 μl of RCA products were singly digested with *Xho*I, *Nco*I, *Sac*I, and *Spe*I. Three microliters of digested and undigested RCA products were subjected to electrophoresis on 1% agarose gel with the GeneRuler^{TM} 1 kb DNA Ladder (Fermentas, Burlington, Canada) and with 2 μl of the control PCR product corresponding to the entire DNA molecule (3640 nts). After electrophoresis, the gels were blotted on the Amersham Hybond-N+ membrane (GE Healthecare, Uppsala, Sweden) according to manufacturer's instructions. The blotted membranes were hybridized with 4 different Dig-labeled DNA probes amplified using the primer pairs listed in Table 2 and the Dig DNA labeling mix (Roche, Basel, Switzerland). Prehybridization and hybridization were carried overnight at 45 °C in the DIG Easy Hyb Granules solution (Roche, Basel, Switzerland) according to manufacturer's conditions. Washing and detection of blotted membranes were done following standard hybridization procedures.

Conventional PCR and quantitative (q) real time PCR assays

Total DNA was extracted using a CTAB-based procedure (Murray and Thompson, 1980) from 150 mg of leaves from all six CCDD-affected TK sources and from two virus-free Eureka lemon seedlings, resuspended in 100 μl of nuclease-free water. The concentration of purified total DNAs was adjusted to *ca.* 100 ng/μl.

Conventional PCR was performed using 1 μl of total DNA in a reaction mixture containing: 1X GoTaq DNA Polymerase buffer (Promega, USA), 0.25 mM each dNTPs, 0.25 μM of each primer (3202fw/6rev, Table 2) and 1U of GoTaq DNA Polymerase (5U/μl) (Promega, Madison, USA). The amplification conditions were: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, 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 visualized by staining with GelRed (Biotium, Hayward, USA). To confirm the specificity of the amplification, the PCR product recovered from TK4 was purified, cloned and sequenced.

Quantitative PCR assays were performed by adding 1 μl of the total DNA to the reaction mix containing 1X PerfeCTa SYBR Green SuperMix (Quanta Bioscienecs, Gaithersburg, USA), 0.32 μM of each primer (1583fw/1650rev, Table 2), in a final volume of 12.5 μl. The cycling profile consisted of 10 min incubation at 95 °C 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 detected amplicons.

The efficiency and sensitivity of the qPCR assay was determined using a 10-fold (from 1 to 10^{-5}) serial dilution, prepared by diluting the TK4 total DNA extract in the total DNA recovered from the healthy Eureka lemon. Standard curves were generated using two replicates of each dilution in two independent assays. To assess the accuracy and reproducibility of the results in the same run or in different runs, the Cq values from different DNA standard dilutions were compared, and intra and inter-assay CV calculated. All assays included positive, negative and non-template controls (NTC).

Acknowledgments

We thank Dr. Federica Cattonaro at IGA, Udine, Italy, for her assistance for deep sequencing.

This study was supported by Fondazione Cassa di Risparmio di Puglia (2010/2011) and by a dedicated grant from the Italian Ministry of Economy and Finance to the National Research Council for the project "Innovazione e Sviluppo del Mezzogiorno - Conoscenze Integrate per Sostenibilità ed Innovazione del Made in Italy Agroalimentare - Legge n. 191/2009".

References

Al Rwahnih, M.A., Daubert, S., Golino, D., Rowhani, A., 2009. Deep sequencing analysis of RNAs from a grapevine showing syrah decline symptoms reveals a multiple virus infection that includes a novel virus. Virology 387, 395–401.

Anonymous, 1978. A whitefly *Parabemisia myricae* (Kuwana) - California. USDA Animal and Hea1th Inspection Service, Cooperative Plant Pest Report 3, 617.

Anonymous, 1986. *Parabemisia myricae* (Kuwana). Distribution maps of pest, Commonwealth Institute of Entomology, map n. 479.

Bensimhon, M.J., Gabarro-Arpa, J., Ehrlich, R., Reiss, C., 1983. Physical characteristics in eucaryotic promoters. Nucleic Acids Res 11, 4521–4540.

Birnstiel, M.L., Busslinger, M., Strub, K., 1985. Transcription termination and 3'processing: the end is in site!. Cell 41, 349–359.

Bisaro, D.M., 1996. Geminivirus DNA replication. In: DNA Replication in Eucaryotic Cells. Cold Spring Harbor Laboratory Press, pp. 833–854.

Bové, J.M., 1995. Virus and Virus-like Disease of Citrus in the Near East Region. FAO Publishing Division, Rome, p. 518.

Breitbart, M., Salamon, P., Andresen, B., Mahaffy, J.M., Segall, A.M., Mead, D., Azam, F., Rohwer, F., 2002. Genomic analysis of uncultured marine viral communities. In: Proceedings of the National Academy of Sciences of the United States of America 99, pp. 14250–14255.

Briddon, R.W., Heydarnejad, J., Khosrowfar, F., Massumi, H., Martin, D.P., Varsani, A., 2010. Turnip curly top virus, a highly divergent geminivirus infecting turnip in Iran. Virus Res. 152, 169–175.

Brown, J.K., Fauquet, C.M., Briddon, R.W., Zerbini, M., Moriones, E., Navas-Castillo, J., 2011. Family Geminiviridae. In: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Vitus taxonomy. Ninth Report of the International Committee on Taxonomy of Viruses, Elsevier-Academic Press, Amsterdam, The Netherlands, pp. 351–373.

Çinar, A., Kersting, U., Önelge, N., Korkmaz, S., Şaş, G., 1993. Citrus virus and virus-like disease in the eastern Mediterranean region of Turkey. In: Moreno, P., da Graça, J.V., Timmer, L.W. (Eds.). Proceedings of the 12th Conference of International Organization of Citrus Virologist, IOCV. Riverside, pp. 397–400.

Çinar, A., Korkmaz, S., Kersting, U., 1994. Presence of new whitefly-borne citrus disease of possible viral aetiology in Turkey. FAO Plant Prot. Bull. 42, 73–75.

Delwart, E.L., 2007. Viral metagenomics. Rev. Med. Virol. 17, 115–131.

Dinsdale, E.A., Edwards, R.A., Hall, D., Angly, F., Breitbart, M., et al., 2008. Functional metagenomic profiling of nine biomes. Nature 452, 629–632.

Edwards, R.A., Rohwer, F., 2005. Viral metagenomics. Nat. Rev. Microbiol. 3, 504–510.

European Food Safety Authority, 2008. Pest risk assessment made by France on Citrus chlorotic dwarf virus considered by France as harmful in the French overseas departments of French Guiana, Guadeloupe, Martinique and Réunion. EFSA J. 684, 1–17.

Fauquet, C.M., Stanley, J., 2003. Geminivirus classification and nomenclature: progress and problems. Ann. Appl. Biol. 142, 165–189.

Garcia Segura, S., Garijo Alba, C., Garcia, Y., Garcia, E.J., 1992. Contribucio al conocimientoy control de Parabemisia myricae (Kuana, 1927) (Insecta: Homoptera: Aleyrodidae) en Màlaga. Bol. San. Veg. Plagas 18, 57–67.

Garrido, A., 1991. Aleurodidos de los citricos espafioles. Levante Agric. 307-308, 44–53.

Giampetruzzi, A., Roumi, V., Roberto, R., Malossini, U., Yoshikawa, N., La Notte, P., Terlizzi, F., Credi, R., Saldarelli, P., 2012. A new grapevine virus discovered by deep sequencing of virus- and viroid-derived small RNAs in *Cv Pinot gris*. Virus Res. 163, 262–268.

Gutierrez, C., 2000. DNA replication and cell cycle in plants: learning from geminiviruses. EMBO J. 19, 792–799.

Howd, D.S., Hartung, J.S., Brlannsky, R.H., 2002. Ultrastructure of Citrus chlorotic dwarf-infected leaves and bark. In: Durán-Vila, N., Milne, R.G., Graça, J.V. (Eds.). Proceedings of the 15th Conference of International Organization of Citrus Virologist, IOCV. Riverside, pp. 373–377.

Inoue-Nagata, A.K., Albuquerque, L.C., Rocha, W.B., Nagata, T., 2004. A simple method for cloning the complete begomovirus genome using the bacteriophage φ29 DNA polymerase. J. Virol. Methods 116, 209–211.

Ito, T., Ieki, H., Ozaki, K., 2002. Simultaneous detection of six citrus viroids and Apple stem grooving virus from citrus plants by multiplex reverse transcription polymerase chain reaction. J. Virol. Methods 106, 235–239.

Kersting, U., Korkmaz, S., Çinar, A., Ertugrul, B., Onelge, N., Garnsey, S.M., 1996. Citrus chlorotic dwarf: A new whitefly-transmitted disease in the east Mediterranean region of Turkey. In: da Graça, J.V., Moreno, P., Yokomi, R. (Eds.). Proceedings of the 13th Conference of International Organization of Citrus Virologist, IOCV. Riverside, pp. 220–225.

Korkmaz, S., Çinar, A., Bozan, O., Kersting, U., 1994a. Distribution and natural transmission of a new whitefly-borne virus disease of citrus in the eastern Mediterranean region of Turkey. In: Proceedings of the 9th Congress of the Mediterranean Phytopathological Union, pp. 437–439.

Korkmaz, S., Çinar, A., Demirer, E., Önelge, N., 1994b. Greenhouse observation on the susceptibility of 36 citrus varieties to a new whitefly-borne virus. In: Proceedings of the 9th Congress of the Mediterranean Phytopathological Union, pp. 305–306.

Korkmaz, S., Çinar, A., Kersting, U., Garnsey, S.M., 1995. Citrus chlorotic dwarf: a new whitefly-transmitted virus-like disease of citrus in Turkey. Plant Dis. 79, 1074.

Korkmaz, S., Garnsey, S.M., 2000. Major virus disease: chlorotic dwarf. In: Timmer, P., Garnsey, S.M., Graham, T. (Eds.), In: Compendium of Citrus Diseases, 2nd Edition Pub. APS Press, pp. 55–56.

Kreuze, J.F., Perez, A., Untiveros, M., Quispe, D., Fuentes, S., Barker, I., Simon, R., 2009. Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: a generic method for diagnosis, discovery and sequencing of viruses. Virology 388, 1–7.

Kuwana, I., 1928. Aleyrodidae or white flies attacking citrus in Japan. Sci. Bull. Min. Agric. Forest. Dept. I, 41–78.

Loconsole, G., Fatone, M.T., Savino, V., 2009. Specific digoxigenin-labelled riboprobes for detection of Citrus psorosis virus and Citrus variegation virus by molecular hybridization. J. Plant Pathol. 91, 311–319.

Loconsole, G., Saponari, M., Savino, V., 2010. Development of real-time PCR based assays for simultaneous and improved detection of citrus viruses. Eur. J. Plant Pathol. 128, 251–259.

Longo, S., Russo, A., Siscaro, G., 1990. Note sui nemici naturali di Parabemisia myricae, un "nuovo" aleirode dannoso agli agrumi in Italia. Inf. Agr. 46, 135–137.

Lopez-Bueno, A., Tamames, J., Velazquez, D., Moya, A., Quesada, A., et al., 2009. High diversity of the viral community from an Antarctic lake. Science 326, 858–861.

Lu, C., Meyers, B.C., Green, P.J., 2007. Construction of small RNA cDNA libraries for deep sequencing. Methods 47, 110–117.

Marja, C.P., Timmermans, O., Prem, Das, Messing, J., 1994. Geminiviruses and their uses as extrachromosomal replicons. Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 79–112.

Martelli, G.P., Yilmaz, M.A., Savino, V., Baloglu, S., Grieco, F., Guldur, M.E., 1996. Properties of a citrus isolate of olive latent virus 1, a new necrovirus. Eur. J. Plant Pathol. 102, 527–536.

Moxon, S., Schwach, F., MacLean, D., Dalmay, T., Studholme, D.J., Moulton, V., 2008. A toolkit for analysing large-scale plant small RNA datasets. Bioinformatics 24, 2252–2253.

Murray, M.G., Thompson, W.F., 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8, 4321–4325.

Muthukumar, V., Melcher, U., Pierce, M., Wiley, G.B., Roe, B.A., 2009. Non-cultivated plants of the Tallgrass Prairie Preserve of northeastern Oklahoma frequently contain virus-like sequences in particulate fractions. Virus Res. 141, 169–173.

Nash, T.E., Dallas, M.B., Reyes, M.I., Buhrman, G.K., Ascencio-Ibañez, J.T., Hanley-Bowdoin, L., 2011. Functional analysis of a novel motif conserved across Geminivirus rep proteins. J. Virol. 85, 1182–1192.

Ng, T.F.F., Manire, C., Borrowman, K., Langer, T., Ehrhart, L., 2009. Discovery of a novel single-stranded DNA virus from a sea turtle fibropapilloma by using viral metagenomics. J. Virol. 83, 2500–2509.

Palmer, K.E., Schnippenkoetter, W.H., Rybicki, E.P., 1998. Geminivirus isolation and DNA extraction. Methods in Molecular Biology 81, Plant Virology Protocols: From Virus Isolation to Transgenic Resistance. In: Foster, G.D., Taylor, S.C. (Eds.), Humana Press Inc., Totowa, NJ, pp. 41–52.

Rapisarda, C., Siscaro, G., Leocata, S., Asaero, C., 1990. Parabemisia myricae, un nuovo aleirode degli agrumi in Italia. Inf. Fitopatol. 12, 25–30.

Rapisarda, C., Siscaro, G., Russo, A., Longo, S., 1991. Primi dati bionomici sul controllo biologico di Parabemisia myricae (Kuwana) in Calabria e Sicilia orientale (Homoptera: Aleyrodoidea). Atti XVI Congresso nazionale italiano di Entomologia Bari - Martina Franca (Ta) 23/28 settembre, pp. 423–427.

Saponari, M., Manjunath, K., Yokomi, R.K., 2008. Quantitative detection of Citrus tristeza virus in citrus and aphids by real-time. J. Virol. Methods 147, 43–53.

Sternlicht, M., 1979. Parabemisia myricae, a new pest in Israel. Hassadeh 59, 1830–1831.

Takahshi, R., 1952. Aleurotuberculatus and Parabemisia of Japan (Aleyrodidae, Homoptera). Misc. Rep. Res. Inst. Nat. Resour. Tokyo 25, 17–24.

Thomas, T.P., Kunta, M., da Graça, J.V., Setamou, M., Skaria, M., 2010. Suppression of phytophthora infection in citrus infected with viroids. HortScience 45, 1069–1072.

Varsani, A., Shepherd, D.N., Dent, K., Monjane, A.L., Rybicki, E.P., Martin, D.P., 2009. A highly divergent South African geminivirus species illuminates the ancient evolutionary history of this family. Virol. J. 6, 36.

Walker, J.E., Saraste, M., Runswick, M.J., Gay, N.J., 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1, 51–945.

Wartig, L., Kheyr-Pour, A., Noris, E., De Kouchkovsky, F., Jouanneau, F., Gronenborn, B., Jupin, I., 1997. Genetic analysis of the monopartite tomato yellow leaf curl geminivirus: roles of V1, V2, and C2 ORFs in viral pathogenesis. Virology 228, 132–140.

Yazdi, H., Heydarnejad, J., Massumi, H., 2008. Genome characterization and genetic diversity of beet curly top Iran virus: a geminivirus with a novel nonanucleotide. Virus Genes 36, 539–545.

Zerbino, D.R., Birney, E., 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 18, 821–829.

Zhang, Y., Singh, K., Kaur, R., Qiu, W., 2011. Association of a novel DNA virus with the grapevine vein-clearing and vine decline syndrome. Phytopathology 101, 1081–1090.