FIG MILD MOTTLE-ASSOCIATED VIRUS, A NOVEL CLOSTEROVIRUS INFECTING FIG

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SUMMARY

The partial genome sequence of a putative new closterovirus with particles *ca*. 2000 nm long, denoted Fig mild mottle-associated virus (FMMaV), was determined. The 6,290 nt long sequence encompasses seven open reading frames (ORFs), i.e. an incomplete ORF1b encoding the putative RNA-dependent RNA polymerase (RdRp), a 25 kDa protein with unknown functions, a 6 kDa protein with putative nucleotide-binding properties, the 63 kDa homologue of the heat-shock protein 70 (HSP70h), a 64 kDa protein, the minor coat protein (CPm) of 26 kDa in size, and the incomplete coat protein (CP). The genome organization of FMMaV is the same as that of members of the genus *Closterovirus.* This taxonomic position was confirmed by comparative analyses of the RdRp, HSP70h and CPm amino acid sequences which clustered FMMaV in a clade comprising members of the genus *Closterovirus* in phylogenetic trees constructed with these sequences. Comparison with Fig leaf mottle-associated virus 1 (FLMaV-1), the other putative fig-infecting closterovirus currently known, showed *ca.* 30% divergence in the HSP70h gene at the amino acid level. In a preliminary survey carried out in southern Italian fig orchards, FMMaV was detected in three different cultivars.

Key words: Fig, *Closterovirus*, RT-PCR, DOP-PCR, cloning and sequencing, phylogenetic analysis.

INTRODUCTION

Fig (*Ficus carica* L.) hosts several filamentous viruses that have always been found in mosaic-affected fig plants (Martelli, 2009). Two of these are putative members of the family *Closteroviridae* denoted Fig leaf mottle-associated virus 1 (FLMaV-1) (Elbeaino *et al*., 2006) and Fig leaf mottle-associated virus 2 (Elbeaino *et al*., 2007), respectively. A third closterovirus-like virus related to Strawberry chlorotic fleck-associated virus (SCFaV) was recently reported from California (Walia *et al*., 2009).

In the course of further studies on fig mosaic, a tree of cv. Dottato bianco from Calabria (southern Italy) (laboratory code: Cal-1) showed light mottling and little or no malformation of the leaves, i.e. symptoms milder that those generally displayed by mosaic-affected fig plants in southern Italy. Electron microscope observations of leaf dips disclosed the presence of long filamentous particles with distinct cross banding, that resembled very much closterovirus virions. However, no amplification was obtained when leaf extracts were assayed by RT-PCR using FLMaV-1 and FLMaV-2 specific primers (Elbeaino *et al*., 2006, 2007). The possibility was therefore investigated that the virus from accession Cal-1, provisionally called Fig mild mottle-associated virus (FMMaV), differed from the known fig-infecting closteroviruses.

MATERIALS AND METHODS

Mechanical transmission. Young symptomatic leaves of accession Cal-1 were ground in a mortar in the presence of 0.05 M phosphate buffer pH 7.2, containing 2.5% nicotine. The inoculum was rubbed onto celite-dusted leaves of *Chenopodium amaranticolor*, *C. quinoa, Nicotiana benthamiana, N. cavicola, N. occidentalis, Gomphrena globosa* and *Cucumis sativus*, which were grown in a glasshouse at *ca*. 24°C and observed for symptom expression.

Extraction of total nucleic acids (TNAs) and double-stranded RNA (dsRNAs). TNAs were extracted from 100 mg of leaf tissue according to Foissac *et al.* (2001). Tissues were ground in liquid nitrogen, homogenized in 1 ml extraction buffer (6 M guanidine isothiocyanate, 0.2 M sodium acetate, 1 M potassium acetate, 0.025 mM EDTA, 2.5% PVP-40), and added to 6 M sodium iodide and 0.15 M sodium sulphite, 150 µl ethanol and 25 µl of a silica particles suspension (1 g

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ml^{-1}, pH 2.0). After stripping by heat treatment in sterile water (70°C for 3 min) and centrifugation for 3 min at 16,000 g, TNAs were recovered and stored at -20°C until use.

dsRNAs were recovered from 15-20 g leaf vein tissues following a phenol/chloroform extraction procedure and chromatography through cellulose CF-11 column in the presence of 17% ethanol, according to Dodds (1993). dsRNAs were further purified by chromatography through a second column of micro-particles cellulose CC41 (Whatman, UK) and subjected to a series of nuclease digestions using RNase-free DNase I (60 µg/ml) and DNase-free RNase A (0.5 µg/ml) (Saldarelli *et al.*, 1994). Finally, dsRNA preparations were passed through Micro Bio-Spin 30 Columns in RNase-free Tris (Bio-Rad Laboratories, USA) to eliminate possible contaminants (resins, polyphenols and polysaccharides). Purified dsRNAs were analyzed in 6% PAGE and silver stained.

RT-PCR, cloning and sequencing. Purified dsRNA extracts were used as templates for viral genome amplification. Three hundred ng of nuclease-treated dsRNA in 9 µl of water were denatured at 95°C for 10 min, were chilled on ice and incubated at room temperature for 10 min in the presence of 0.5 µg of random hexamers and 1 µl 100 mM methyl mercuric hydroxide (Saldarelli *et al.*, 1994). The methyl mercuric hydroxide was inactivated by adding 1 µl 0.5 M 2-β-mercaptoethanol. Denatured dsRNA were reverse transcribed for 1 h at 39°C by adding a cDNA mixture containing 200 units of Superscript III-reverse transcriptase enzyme (Invitrogen, USA) in a final volume of 20 µl.

To collect preliminary information on the viral sequence present in accession Cal-1, two strategies were used. DOP-PCR was first used to generate contiguous internal sequence data. Amplifications were done using primer DOP4 (5'-CCGACTCGAGNNNNNNTTTACG-3') (Rott and Jelkmann, 2001) from the commer-

Table 1. Nucleotide sequences of specific and degenerate primers used in RT-PCR for amplifying the FMMaV genome. Primers LM3s/LM3a, were used for FMMaV detection in fig orchards.

Primers name	Primers sequence (5' to 3')	Genome location
RdRp4s	GGWGSTKCHAAYACHTGG	1-18
P25a	CTTATCAGCTCGAGGGATGC	713-732
Motif Is	GGITTIGAITTYGGIACIAC	1806-1826
Motif IIa	RTCIAAIGTICCICCICCRAA	2401-2421
P25s	CTTTCTTTTCGGAAGCGTTG	1139-1158
M1a	AGCCTACGTACCGCTTGAGA	1993-2012
M1s	TGCTGACACGTTCGATCTTC	2134-2153
P70a	TTGATCCAGTCCGAAAGAGC	2507-2526
P70s	CCGCAGAATTGGTATTACGG	3480-3499
P90a	TGACGTAACTCGAGGGGAAC	4196-4215
P90s	CGTTCGAGGGAACTATCTGC	4977-4996
CPa	GGTCTGGCACAGGAGTAGGA	6006-6025
LM3s	AAGGGGAATCTACAAGGGTCG	3075-3095
LM3a	TATTACGCGCTTGAGGATTGC	3366-3386

(s: sense), (a: antisense), I (Inosine), (R: A+G), (Y: T+C), (S: C+G), (K: G+T), (H: A+T+G) and W(A+T).

cial kit "DOP PCR Master", following the manufacturer's instructions (Roche, Switzerland). RT-PCR followed using Tian *et al*. (1996) set of degenerate sense and antisense primers for the specific amplification of motif I and II of closteroviral HSP70h sequence (Tab. 1).

A further primer set was designed for the specific amplification of the 3' terminal portion of the FMMaV polymerase gene. The degenerate sense primer, denoted RdRp4s, was constructed with a high level of nucleotide degeneracy, based on alignment of conserved motif of the RdRp of *Citrus tristeza virus* (CTV), *Beet yellows virus* (BYV) and *Grapevine leafroll-associated virus 2* (GLRaV-2). This primer was used together with an antisense-specific primer (P25a), designed on a clone previously obtained with DOP-PCR (Tab. 1). All PCR amplifications were performed on a random-primed reverse-transcribed dsRNA template. The PCR mixture consisted of 1 mM $MgCl_2$, 0.4 mM dNTPs, 0.2 and 0.6 µM of primers P25a and RdRp4s, respectively, and 1 unit of Taq polymerase (Promega, USA). PCR consisted of 35 cycles, with an initial denaturing temperature of 94°C for 1 min, 48°C for 1 min and an elongation time of 2 min. All PCR primers designed on the 5' and 3' termini of each DOP-generated clones to close all sequence gaps are reported in Tab. 1.

Amplification strategy and clone position on the FMMaV genome are shown in Fig. 1. All amplicons were transformed in pGEM-T easy vector (Promega, USA) and/or in StrataCloneTM PCR Cloning vector pSC-A (Stratagene, USA), according to their length, cloned into *Escherichia coli* DH5α or SoloPACK cells, and automated sequenced (Primm, Italy).

Detection of FMMaV in fig orchards. To assess the distribution of FMMaV in the field, a small-scale survey was carried out in July 2008 in a fig-growing area of Calabria. Sixty samples were collected in three orchards from plants of cvs Dottato bianco, Brogiotto nero and Severone precoce. PCR for FMMaV detection was conducted on reverse-transcribed TNAs extracted from leaf vein tissues, using a primer set (LM3s/a) designed on the HSP70h sequence (Tab. 1). PCR consisted of 35 cycles, with denaturation at 94°C for 30 sec, annealing at 58°C and 30 sec elongation. Limitedly to the FMMaV-positive samples, PCR assays were extended for detection of FLMaV-1, FLMaV-2, Fig mosaic virus (FMV) (Elbeaino *et al*., 2009) and Fig latent virus 1 (FLV-1) (Gattoni *et al*., 2009) in the hope to identify trees with single FMMaV infections. dsRNA analysis completed the sanitary assessment of these plants.

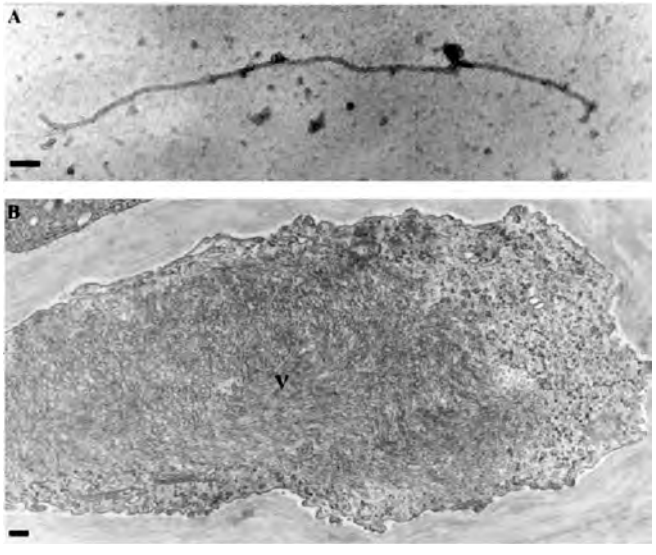

Fig. 1. A. A virus particle in a dip from a symptomatic fig leaf. Bar = 100 nm. B. A large aggregate of filamentous virus particles (V=Virus-like) in a phloem companion cell. Bar = 250 nm.

Computer-assisted analysis. Nucleotide and protein sequences were analysed with the assistance of the DNA Strider 1.1 program (Marck, 1988). Multiple alignments of nucleotide and amino acid sequences were obtained using the default options of CLUSTALX 1.8 (Pearson and Lipman, 1988). Search for homologies with proteins from the protein information resources database (PIR, release 47.0) was done with FASTA (Pearson and Lipman, 1988), BlastX and BlastN (Altschul *et al*., 1990) programmes. Phylogenetic trees were constructed using the NJPLOT package (Perrière and Gouy, 1996) with 1000 bootstrap replicates. Protein analysis for hydrophobicity and presence of transmembrane domains were conducted with the use of DNA Strider 1.1 and "DAS"- transmembrane prediction server (Cserzo *et al*., 1997), respectively.

Electron microscopy. Dips in 2% aqueous uranyl acetate were made from leaf petioles of accession Cal-1. For thin sectioning, tissue pieces from veins and mesophyll tissues of the discoloured areas of young leaves were processed according to standard procedures at 4°C throughout (Martelli and Russo, 1984), i.e. fixation in 4% glutaraldehyde in 0.05 M phosphate buffer for 2 h, post-fixation in 1% osmium tetroxide for 2 h, staining overnight in 2% aqueous uranyl acetate, dehydration in graded ethanol dilutions and embedding in TAAB low viscosity resin**.** Thin sections were stained with lead citrate and viewed with a Philips Morgagni 282D electron microscope.

RESULTS AND DISCUSSION

Mechanical transmission. No virus was recovered with any of several mechanical transmission attempts. All inoculated hosts remained symptomless and no virus was apparently transferred from any of them by sub-inoculation to new sets of herbaceous plants. All indicators were PCR-negative when checked for the presence

of FMMaV. Thus, FMMaV behaved like FLMaV-1 and FLMaV-2 as a presumably untransmissible virus onto herbaceous hosts (Elbeaino *et al*., 2006, 2007).

Electron microscopy. Filamentous particles with distinct cross banding and a length of up to *ca*. 2000 nm were seen in dips from symptomatic Cal-1 leaves (Fig. 1A). Massive accumulations of filamentous elements, interpreted as profiles of virus particles, were present in some differentiating sieve elements and phloem companion cells (Fig. 1B), resembling very much a condition common to closteroviral infections (Martelli and Russo, 1984). No FMV particles (= double-membrane bodies) (Elbeaino *et al*., 2009) were seen in mesophyll cells.

dsRNA analysis. Electrophoretic analysis of Cal-1 leaf vein tissue extracts yielded a dsRNA band of *ca.*19 kb, a size similar to that of dsRNAs recovered from plants infected by some members of the family *Closteroviridae*. In a comparative electrophoretic run, dsRNAs from FMMaV, FLMaV-1 and FLMaV-2 migrated close to one another. FLMaV-2 dsRNA, however, migrated slightly faster than those of the other two viruses, in line with the notion that FLMaV-2 has presumably a smaller genome as several members of the genus *Ampelovirus* do (Martelli *et al*., 2005).

DOP and RT-PCR generated clones. DOP-PCR run on reverse-transcribed FMMaV dsRNA template yielded products ranging from 350 to 1200 bp in size. After cloning and sequencing of the largest DOP-generated amplicons four clones (DOP25, DOP70, DOP90 and DOPCP) proved to be of viral origin. Blast analysis disclosed that these clones shared amino acid homology with the RNA-dependent RNA polymerase (RdRp), the heat-shock protein 70 analogue (HSP70h), the p61 and the coat protein (CP) sequences of CTV. The set of primers designed by Tian *et al*. (1996) on the conserved motif I and II of the HSP70h gene generated an amplicon of 620 bp. The obtained clones were positioned in an order suggested by the organisation of CTV genome, then sense and antisense virus-specific primers were used in RT-PCR for filling sequence gaps between clones (Fig. 2). The RdRp4s degenerate primer designed in this work amplified an additional part of the RdRp gene of FMMaV when used in conjunction with P25a primer. In the end, a continuous sequence of 6,290 nt in size was obtained, spanning the FMMaV genome area between the incomplete RdRp and CP genes (Fig. 2). This sequence was deposited in GenBank under the accession number FJ611959.

Sequence analyses and function of FMMaV-encoded proteins. ORF1b (783 nt) has a sequence truncated at the 5' terminus and ends at nucleotide position 781-783 with an amber codon (UAG). The highest identity of this sequence at the amino acid (aa) level did not exceed 50% with comparable sequences of six species of the

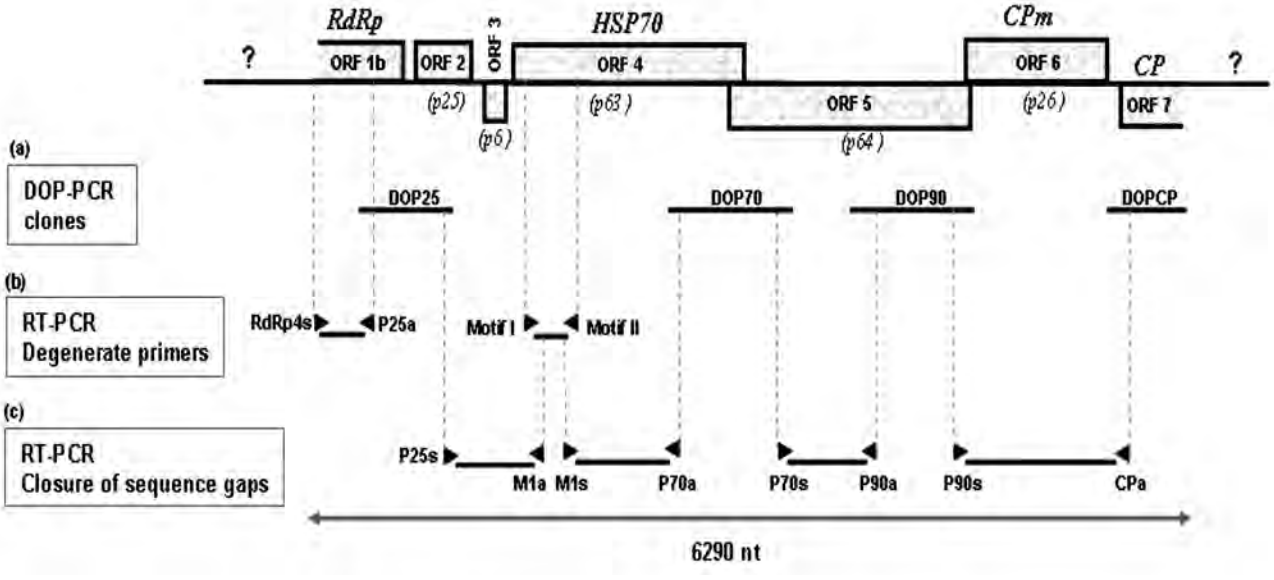

Fig. 2. Schematic representation of the cloning strategy and location of cDNA clones on FMMaV genome. Boxed regions correspond to ORFs and boxes with open ends correspond to incomplete ORFs. The putative function of proteins and their estimated molecular weights are reported on and above the boxes. (a) DOP-PCR-generated clones are positioned sequentially according to Blast analysis. (b) RT-PCR-generated clones obtained with the use of degenerate primers (RdRp4s, Motif Is and Motif IIa). (c) Sequence gaps between the clones are closed with the use of sense and antisense virus-specific primers (named P and indicated by arrow heads) designed on different DOP- and RT-PCR-generated clones. A total of 6290 nucleotides of the FMMaV genome were identified.

Table 2. Amino acid identity (%) of three gene products, RdRp (bold), HSP70 (italics) and CPm (shadowed), between FMMaV and some members of the genus *Closterovirus*; Fig mild mottle-associated virus (FMMaV); *Citrus tristeza virus* (CTV); *Beet yellows virus* (BYV); *Beet yellow stunt virus* (BYSV); Strawberry chlorotic fleck-associated virus (SCFaV); Raspberry mottle virus (RMoV); *Grapevine leafroll-associated virus 2* (GLRaV-2)

Virus species	FMMaV	CTV	BYV	BYSV	SCFaV	RMoV	GLRaV-2
		50	**35**	**36**	**50**	**49**	**39**
FMMaV	**100**	*36*	*35*	*35*	*35*	*38*	*32*
		22	15	17	7	22	16
			49	**52**	**60**	**59**	**51**
CTV		**100**	*41*	*40*	*47*	*45*	*40*
			20	23	13	36	22
				60	**49**	**52**	**57**
BYV			**100**	*52*	*40*	*43*	*47*
				38	**10**	**23**	**37**
					55	**53**	**60**
BYSV				**100**	*40*	*39*	*47*
					11	**26**	**23**
						61	**49**
SCFaV					**100**	*52*	*38*
						13	**10**
							48
RMoV						**100**	*39*
							24
GLRaV-2							**100**

genus *Closterovirus* (Tab. 2). Clustal alignment of amino acid sequences of FMMaV polymerase with those of the same six closteroviruses starting from motif V contained the highly conserved regions reported in the literature (Koonin, 1991; Koonin and Dolja, 1993; Karasev *et al.*, 1994) , plus two previously unreported highly conserved stretches of amino acids located downstream of motif VIII, denoted motif IX and X (Fig. 3A).

An intergenic region of 157 nt separates ORF1b from ORF2 (666 nt in size), which initiates with a start codon at position 940 and ends with an opal stop codon (UGA) at position 1603-1605. ORF2 potentially encodes a 222 aa polypeptide with an estimated mol. mass of 25 kDa (p25), which did not show significant aa sequence similarity with any other protein present in database.

ORF3 (171 nt) initiates at position 1610 and terminates with an ochre codon (UAA) at position 1780. This protein has a predicted mol. mass of 6 kDa, and shares the highest identity (38%) with the p6 hydrophobic protein encoded by CTV ORF3. As predicted, this ORF constitutes a hydrophobic protein and the deduced amino acid sequence includes a stretch of non polar amino acids, which is presumed to form a transmembrane helix encompassing 26 aa starting from position 8 to 33. In the genus *Closterovirus*, these small-sized hydrophobic proteins, thought to have membrane-binding properties (Dolja *et al.*, 1994), occur only in some species such as CTV, BYV and BYSV (Fig. 3B).

ORF4 (1767 nt) begins at position 1793 and ends at position 3557-3559 with a UGA stop codon. It encodes a polypeptide with a mol. mass of 63 kDa (p63) that contains all the eight conserved sequence motifs typical of the N-terminal half of the HSP70h gene of *Closteroviridae* genomes (Koonin, 1991; Koonin and Dolja, 1993; Poche *et al.*, 1989; Agranovsky *et al.*, 1991). The FMMaV HSP70h sequence had the highest identity at the aa level (38%) with the comparable sequence of Raspberry mottle virus (RMoV) (Tab. 2).

In course of this study, an additional fragment (653 nt) of the FLMaV-1 HSP70h gene was cloned and sequenced and assembled with the previously reported 616 nt sequence (accession No. AM113547, Elbeaino *et al.*, 2006) so as to obtain a sequence 1269 nt in size. This sequence was used in the alignment for comparing the HSP70h genes of FMMaV and FLMaV-1, the only putative member of the genus *Closterovirus* known to infect fig trees. HSP70h sequences of the two viruses diverged by 30% at the aa level, a value that supports their classification as different species.

ORF5 (1683 nt) overlaps ORF4 by 124 nt, begins at position 3435, terminates at position 5117 and codes for a protein with mol. mass of 64 kDa (p64) which, in the family *Closteroviridae*, is always present downstream the HSP70h gene. This product had 28% identity at the aa level with comparable proteins of CTV, BYSV and *Beet*

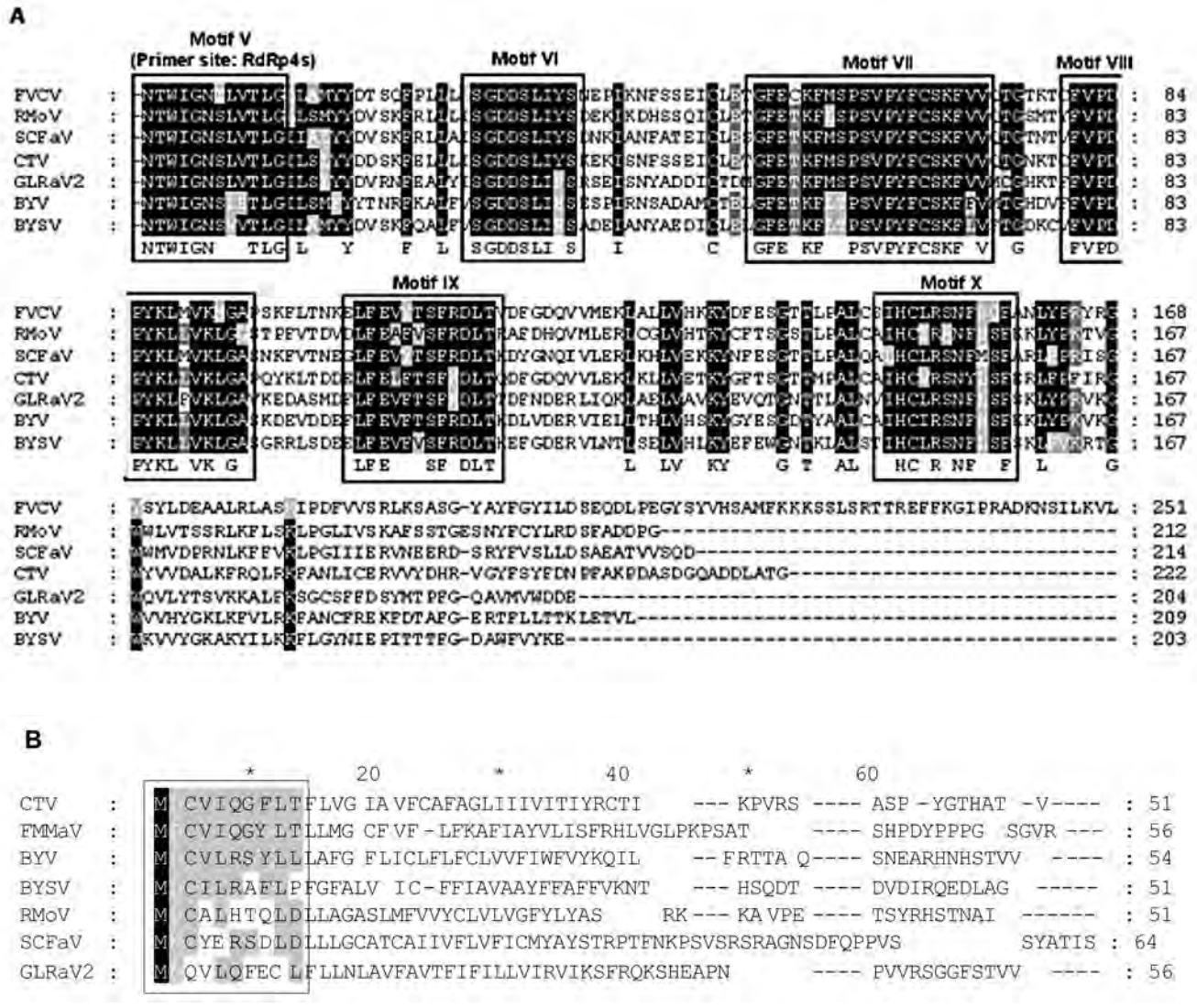

Fig. 3. A. Amino acid sequence alignment of RdRp genes of FMMaV and of other members of the *Closterovirus* genus. In addition to the conserved motifs V to VIII, two additional motifs, denoted IX and X, were identified. All motifs are boxed. B. Amino acid sequence alignment of the small hydrophobic proteins (p6) of FMMaV, CTV, RMoV, BYV, BYSV, GLRaV2 and SCFaV.

yellows virus (BYV).

ORF6 (702 nt) overlaps ORF5 by 51 nt, starts at position 5066 and ends at position 5767. The predicted mol. mass of ORF6 expression product is a 26 kDa (p26) polypeptide, a size in line with that of other closteroviral proteins located downstream of ORF5. The highest similarity disclosed by blast analysis was with CPm of CTV and RMoV, with aa identity of 22% (Tab. 2).

An intergenic region of 174 nt separates ORF7 from ORF8. This last incompletely sequenced ORF (349 nt) initiates at position 5941 and shares 19% aa sequence homology with the CP of CTV.

Taxonomic position and putative genome organisation of FMMaV. In phylogenetic trees constructed with the amino acid sequences of RdRp, HSP70h and CPm of known members of the family *Closteroviridae*, FMMaV consistently clustered in a clade comprising representatives of the genus *Closterovirus* (Fig. 4), thus qualifying as a putative species of this genus. The molecular information gathered, the size of both dsRNA and virus particles suggest that FMMaV may possess a genome organisation and a number of ORFs more similar to that of CTV or BYSV than BYV, three species that exemplify the differential genome structure found in the genus *Closterovirus* (Koonin, 1991). This likelihood is supported by the presence in FMMaV of ORF2, a cistron encoding a 25 kDa protein present also in CTV (p30) and BYSV (p30), but not in BYV. If one considers that, in addition to the seven genes already identified, there is one (ORF1a) at the extreme 5' end and that, by analogy with known closteroviral genomes, the presence of at least other two (as in BYSV) or four (as in CTV) ORFs can be expected at the 3' end, FM-

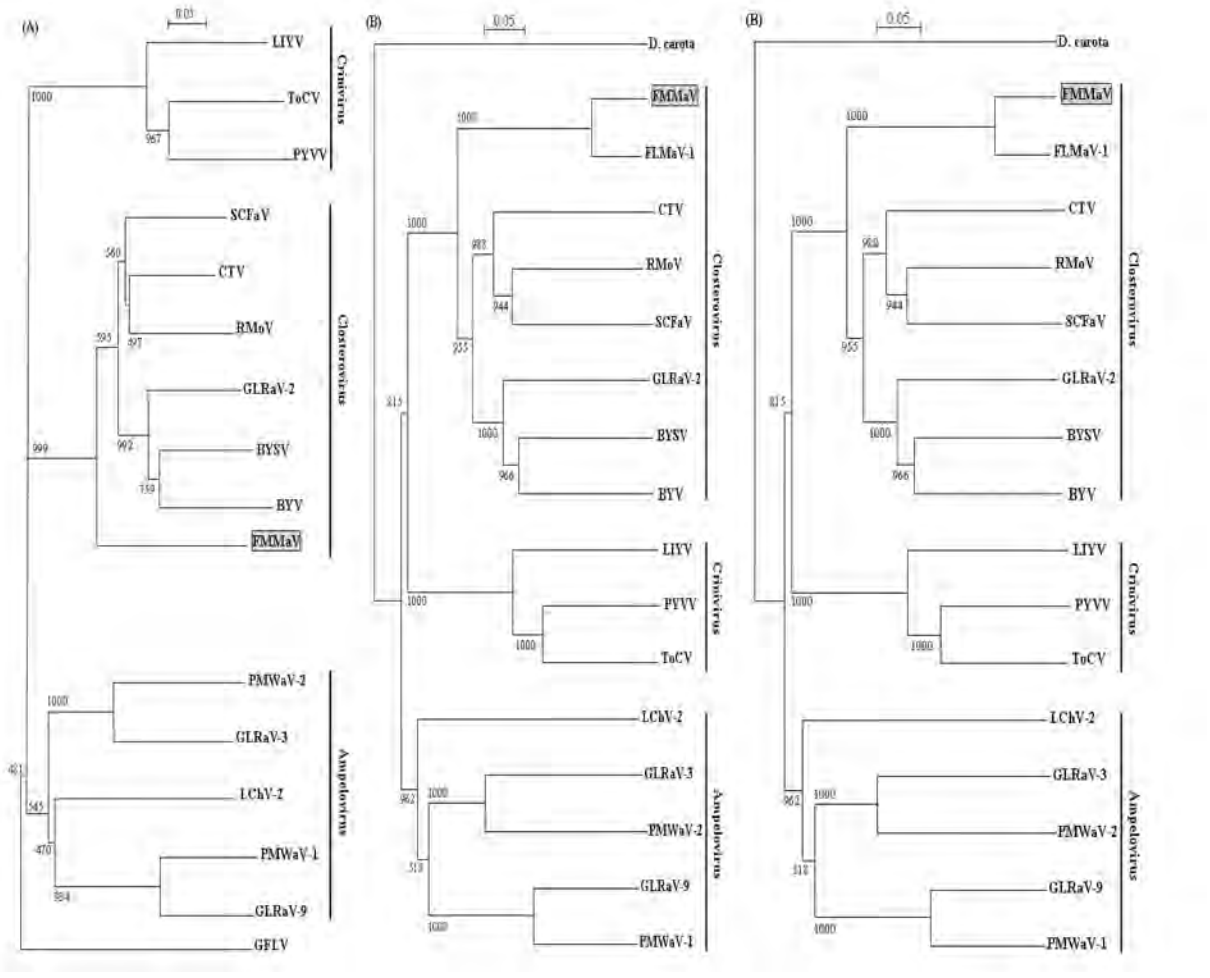

Fig. 4. Phylogenetic trees constructed with RdRp (A), HSP70 (B), and CPm/CP (C) sequences. In all trees, FMMaV groups in the branch comprising members of the genus *Closterovirus. Grapevine fanleaf virus* (GFLV, NC-003203) was used as a non-closterovirus outgroup in the RdRp tree, while carrot (*D. carota,* X53852) HSP70 sequence was used as an outgroup in the HSP70h and CPm trees. The bootstrap values (1000 replicates) are shown at each node. Criniviruses: ToCV (*Tomato chlorosis virus,* NC-00734*);* LIYV (*Lettuce infectious yellows virus,* NC-003618); PYVV (*Potato yellow vein virus* NC-006063); Closteroviruses: CTV (*Citrus tristeza virus,* NC-001661); GLRaV-2 (*Grapevine leafroll-associated virus 2,* NC-007448); BYV (*Beet yellow virus,* NC-001598); BYSV (*Beet yellow stunt virus,* U51931); RMoV (Raspberry mottle virus, NC-008585); SCFaV (*Strawberry chlorotic fleck-associated virus,* NC-008366); FMMaV (Fig mild mottle-associated virus, FJ611959); FLMaV-1 (Fig leaf mottle-associated virus *1,* AM113547); Ampeloviruses: PMWaV-1 (*Pineapple mealybug wilt-associated virus 1,* NC-010178); PMWaV-2 (*Pineapple mealybug wilt-associated virus 2,* AF283103); GLRaV-3 (*Grapevine leafroll-associated virus 3,* NC-004667); GLRaV-9 (*Grapevine leafroll-associated virus 9,* AY297819); LChV-2 (*Little cherry virus 2,* NC-005065)

MaV genome could comprise either 10 or 12 ORFs like BYSV or CTV, respectively.

Detection of FMMaV in fig orchards. The survey conducted on 60 trees of three different fig cultivars from southern Italy identified eight FMMaV-positive accessions (13.3%) (Fig. 5). The primers designed on the FMMaV HSP70 gene proved to be virus-specific. In fact, PCR-generated amplicons had 97-100% nucleotide identity with FMMaV sequence. Only three samples were infected by FMMaV only. Symptoms shown by the singly FMMaV-infected plants were comparable to those observed on Cal-1, but whether the mild mottling condition (as opposed to much stronger mosaic and leaf deformations shown by most mosaic-

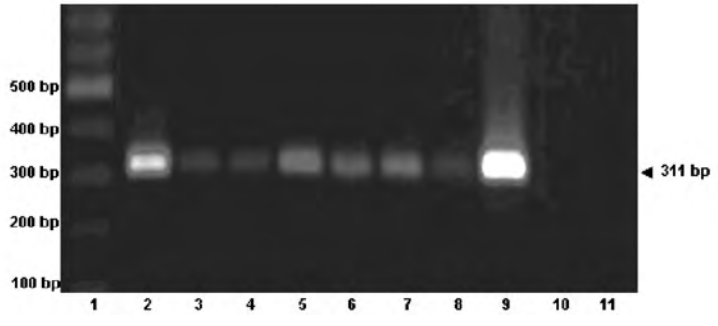

Fig. 5. RT-PCR electropherogram of RT-PCR amplicons (311 bp) from fig trees obtained using the FMMaV-specific primer set LM3s/LM3a. Lanes 2 to 8, FMMaV-infected samples; Lane 9, positive control. Lanes 10 and 11, FMMaV-negative samples. Lane 1: DNA marker.

diseased fig trees) can be regarded as typically induced by FMMaV remains to be determined.

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