

## IDENTIFICATION OF A SECOND MEMBER OF THE FAMILY *CLOSTEROVIRIDAE* IN MOSAIC-DISEASED FIGS

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### SUMMARY

A double-stranded RNA (dsRNA) *ca.* 19 kb in size was recovered from a fig tree of Algerian origin (F3) showing chlorotic mottling of the leaves and clearing of the second and third veins. Filamentous closterovirus-like particles up to 2100 nm in length and with distinct cross banding were observed in leaf dips. Cytopathology of thin sectioned phloem cells was similar to that elicited by members of the family *Closteroviridae*. RT-PCR assays with primers specific to the recently described Fig leaf mottle-associated virus (FLMaV) were negative. No virus was recovered by mechanical inoculation to herbaceous hosts. Using denatured F3 dsRNA preparations as template, a 625 bp cDNA fragment was amplified by RT-PCR using degenerate primers designed on the phosphate motifs 1 and 2 of the heat shock-protein 70 homologue (HSP70) of members of the family *Closteroviridae*. Sequence analysis demonstrated that the sequenced 625 bp fragment was part of a closteroviral HSP70 gene, with sequence identity to FLMaV of *ca.* 45% and 28% at the nucleotide and amino acid level, respectively. Comparison with the HSP70 amino acid sequences of other closteroviral species showed homologies of 48%, 37% or 36% with *Little cherry virus 2* (LChV-2), *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Pineapple mealybug wilt-associated virus 2* (PMWaV-2), respectively. In a phylogenetic tree constructed with the amino acid sequences of the HSP70 genes of several members of the family *Closteroviridae*, isolate F3 clustered with members of the genus *Ampelovirus*. In RT-PCR assays with specific primers designed to match the F3 HSP70 sequence, 4 of 57 field-grown Italian figs trees yielded amplicons of the expected size, while Multiplex-PCR proved effective for the simultaneous detection of double infections by FLMaV and isolate F3. The different sizes of dsRNA and virus particle lengths of F3 and FLMaV, the low sequence homology of the F3 HSP70 gene with that of

FLMaV, and the different phylogenetic affiliation constitute evidence that isolate F3 belongs to a virus species different from FLMaV, for which the name of Fig leaf mottle-associated virus 2 (FLMaV-2) is proposed.

*Key words:* *Ficus carica*, *Closteroviridae*, *Ampelovirus*, dsRNA, HSP70, sequencing, RT-PCR, Multiplex-PCR.

### INTRODUCTION

A fig (*Ficus carica*) accession (coded F3) of unknown variety imported several years ago from Algeria and since grown under screen on the premises of the Mediterranean Agronomic Institute of Valenzano-Bari (Italy), showed recurrent chlorotic mottling of the leaf blade and conspicuous clearing of the second and third order veins. These symptoms are similar to those reported for fig mosaic disease (Condit and Horne, 1933) and resemble those observed on a southern Italian fig tree infected by Fig leaf mottling-associated virus (FLMaV, Elbeaino *et al.*, 2006), a tentative species of the genus *Closterovirus*.

Although electron microscope observations of leaf dips from the Algerian fig tree showed the presence of closterovirus-like particles, repeated RT-PCR assays using FLMaV primers (Elbeaino *et al.*, 2006) gave negative results. This suggested that accession F3 could host a virus different from FLMaV and prompted the investigations reported in this paper.

### MATERIALS AND METHODS

**Mechanical transmission.** Young leaves of accession F3 were triturated in a mortar and pestle in 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 kept in a glasshouse at *ca.* 24°C and observed for symptom expression.

**Total nucleic acid and double-stranded RNA extraction.** Total nucleic acids (TNA) were extracted from about 100 mg of leaf veins homogenized in 1 ml grinding

buffer (4.0 M guanidine isothiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc pH 5.0 and 2.5% w/v PVP-40), and purified according to Foissac *et al.* (2001). For double-stranded RNA (dsRNA) recovery, about 30 g of leaf tissue were ground in a mortar with liquid nitrogen and extracted with two rounds of phenol-chloroform treatment (Dodds, 1993). After CF11 cellulose chromatography, single-stranded nucleic acids were digested as described by Saldarelli *et al.* (1994). Preparations of each nuclease digestion step were incubated for 30 min at 37°C. After phenol/chloroform extraction, and centrifugation at 9,000 g for 10 min, dsRNA was ethanol-precipitated from the supernatant fraction and electrophoresed in 5% TAE polyacrylamide gel (Sambrook *et al.*, 1989) together with dsRNA from a grapevine infected by *Grapevine leafroll-associated virus 2* (GLRaV-2) and from the fig infected by FLMaV.

**Cloning and sequencing.** Nuclease-treated dsRNA pellets were eluted, denatured by boiling at 95°C for 10 min followed by fast cooling, then by the addition of 0.5 µl methyl mercuric hydroxide (100 mM) and, after 10 min at room temperature, the addition of 1 µl of 0.5 M 2-mercaptoethanol. TNA extracts used for field survey and Multiplex-PCR were denatured only by heating. Both TNA and dsRNA extracts were reverse transcribed for 1 h at 39°C by adding 4 µl M-MLV buffer 5x (50 mM tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>), 2 µl of 0.1 M dithiothreitol, 0.5 µl of 10 mM dNTPs, and 200 units of M-MLV reverse transcriptase (Bethesda Research Laboratories, MD, USA) in a final volume of 20 µl. Aliquots of cDNA were PCR-amplified using a set of degenerate primers specific for the closteroviral heat shock protein 70 homologue gene (HSP70) (Tian *et al.*, 1996). Amplicons were electrophoresed in 6% polyacrylamide slab gels, ligated directly into pGEM-T Easy (Promega Corp, Madison, WI, USA) according to the manufacturer's instructions and used to transform *E. coli* DH5a cells. DNA from clones was subjected to automated sequencing (MWG, Biotech, Ebersberg, Germany).

**Computer-assisted analysis of nucleotide and protein sequences.** Nucleotide and protein sequences were assembled using the Strider 1.1 program (Marck, 1988). Protein sequences were aligned by using the Clustal X program (Thompson *et al.*, 1997). Homology with known proteins from the Protein information resources (PIR, release 47.0) was determined using the FASTA (Pearson and Lipman, 1988) and BLASTA (Altschul *et al.*, 1990) programs. Phylogenetic trees were constructed and bootstrap analysis was made with the NEIGHBOR, SEQBOOT, PROTDIST and CONSENSE programs of the PHYLIP package (Felsenstein, 1989).

**Electron microscopy.** Dips in 2% aqueous uranyl acetate were made from leaf petioles of accession F3. 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 0.5% aqueous uranyl acetate, dehydration in ethanol and embedding in low viscosity resin (Agar Scientifics, Stansted, UK) Thin sections were stained with lead citrate and viewed with a Philips Morgagni 282D electron microscope. Controls consisted of leaf tissues from a healthy fig seedling processed as above.

**Primer selection for RT- and Multiplex-PCR.** Sequence data were used to design primers specific for the phosphate motifs 1 and 2 of F3 HSP70 nucleotide sequence i.e. primers F3-s: 5'-GAACAGTGCCTATCAGTTTGATTTG-3' and F3-a: 5'-TCCCACCTCCTGCGAAGCTAGAGAA-3', that amplified a DNA fragment of ca 360 bp in RT-PCR. The cDNA was suspended in 2.5 µl of 10x *Taq* polymerase buffer (Promega Corporation, Madison, WI, USA) containing MgCl<sub>2</sub> to a final concentration of 1 mM, 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 µM of each of the above mentioned specific primers, and 0.2 µl *Taq* polymerase (5 unit/µl) in a final volume of 25 µl.

PCR was carried out in a thermal cycler (Perkin-Elmer 7600), programmed for one cycle at 94°C for 4 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 40 sec. Final extension was at 72°C for 7 min.

For Multiplex-PCR, specific and degenerate primers were designed on the F3 HSP70 nucleotide sequence (present work, accession n° AM286422) and the comparable sequence of FLMaV (Elbeaino *et al.*, 2006, accession n° AM113547). An antisense degenerate primer 5'-GCYGCSSWYGGTTCGTTGA-3', where Y=C+T, S=C+G and W=A+T, was used in conjunction with the sense FLMaV-specific primer "N17-4-s": 5'-CGTGGCTGATGCAAAGTTTA-3' (Elbeaino *et al.*, 2006), and the newly designed sense F3-specific primer "Multi F1-s": 5'-TATCAAGTCGCAATAGGGGGAA-3'. These primers amplified 420 bp and 240 bp DNA fragments from FLMaV and F3 sequences, respectively.

Multiplex-PCR was done under the same conditions and cycling described above for F3 specific detection. Amplification products were resolved in 6% polyacrylamide slab gels.

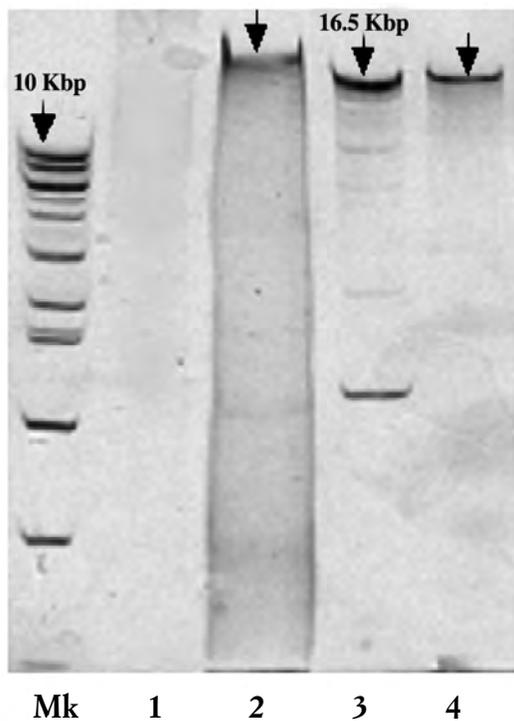
## RESULTS AND DISCUSSION

**Mechanical transmission.** No virus apparently was recovered with any of several mechanical transmission trials. All inoculated hosts remained symptomless and no virus was transmitted from any of them by sub-inoculations to new sets of healthy plants.

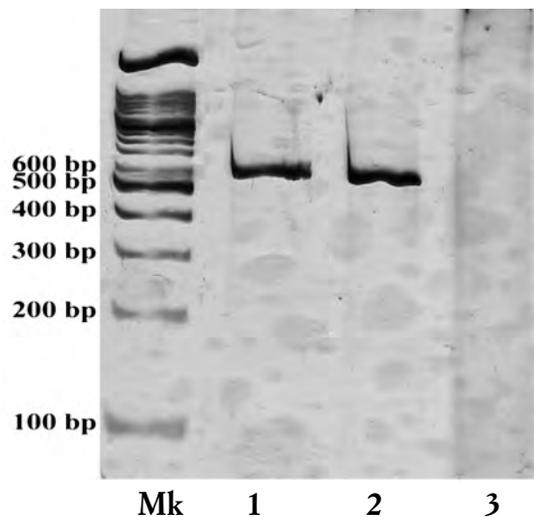
**dsRNA analysis.** Electrophoretic analysis of extracts from F3 leaf tissues consistently yielded a dsRNA band of *ca.* 19 kbp (Fig. 1), larger in size than dsRNAs of GLRaV-2 and FLMaV. Based on the results of several comparative electrophoretic runs, the size of FLMaV RNA previously reported to be *ca.* 19 kbp (Elbaino *et al.*, 2006) was re-estimated as *ca.* 17.5-18 kbp.

**RT-PCR.** Preliminary RT-PCR assays on leaf extracts of symptomatic F3 fig accession using FLMaV-specific primers (Elbeaino *et al.*, 2006) gave negative results. However, when assays were made on the same F3 source material and on leaf extracts from FLMaV-infected fig leaves with degenerate primers (Tian *et al.*, 1996), a DNA fragment of *ca.* 625 bp was amplified from both infected sources (Fig. 2). This was taken as an indication that the fig accession F3 was likely to be infected by a member of the family *Closteroviridae* different from FLMaV.

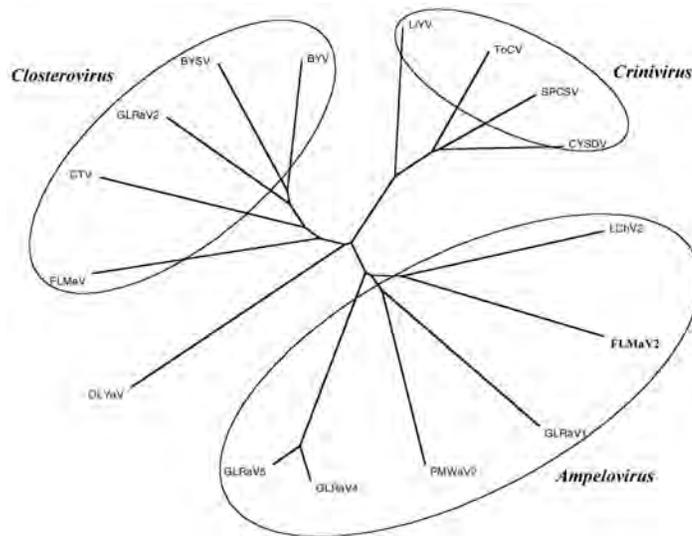
**Identification of the HSP70 gene.** BLAST analysis of the cloned F3 cDNA fragment sequence (625 bp) showed that it belonged to an incomplete open reading frame (ORF) encoding a polypeptide of *ca.* 208 amino acids with *ca.* 45% sequence identity at the nucleotide level with the HSP70 gene of FLMaV (not shown).



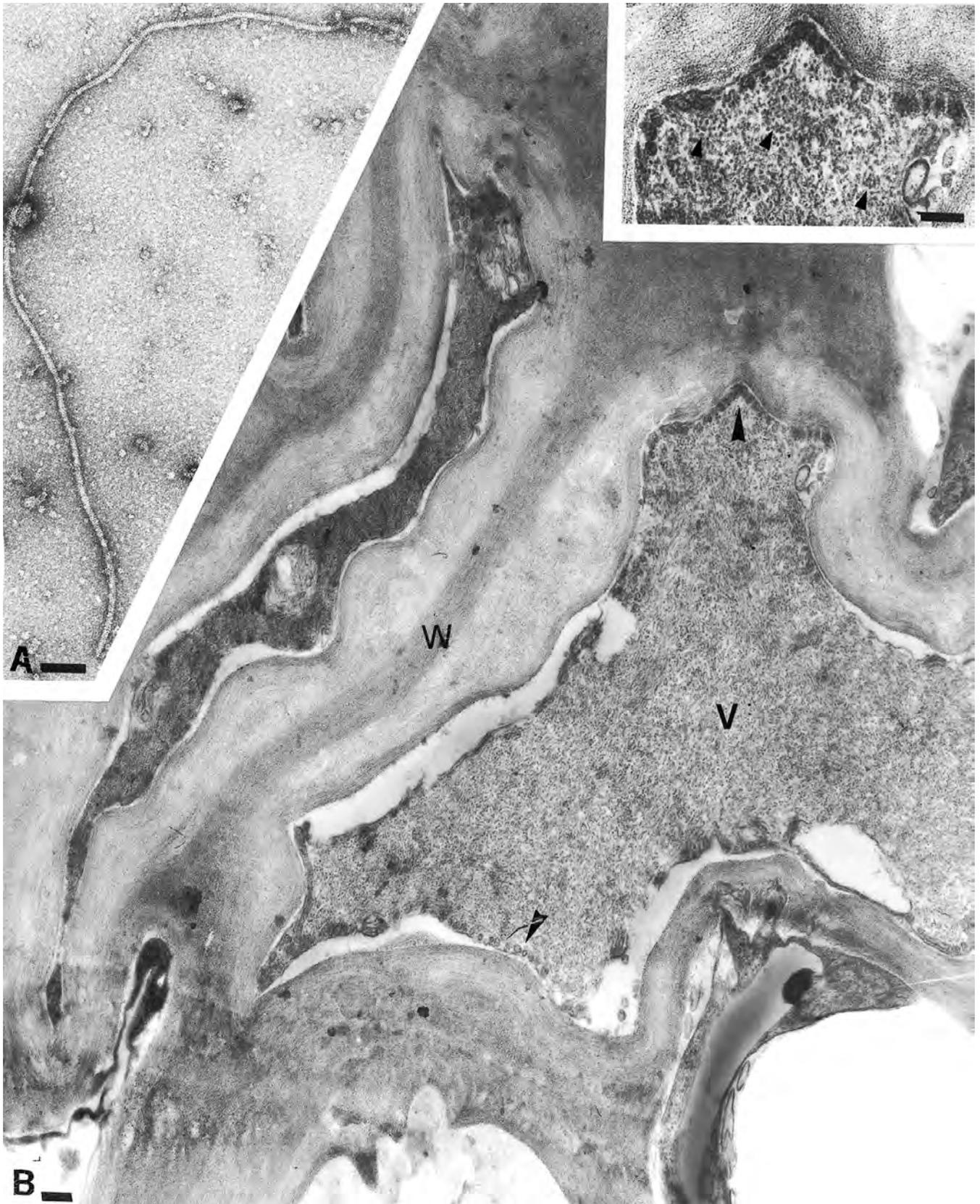
**Fig. 1.** Electrophoretic patterns of dsRNAs extracted from leaf tissues of the Algerian fig accession F3 (lane 2), cortical scrapings from grapevines infected with *Grapevine leafroll-associated virus 2* (GLRaV-2) (lane 3), and leaf tissues of the Italian fig accession N17 infected by Fig leaf mottle-associated virus (FLMaV) (lane 4). Mk, DNA ladder; lane 1, leaf extract from a symptomless fig seedling.



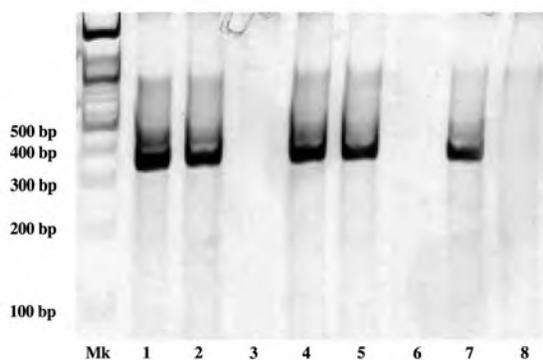
**Fig. 2.** RT-PCR amplification of a *ca.* 625 bp DNA fragment from tissue extracts of fig accessions F3 (lane 1) and N17 infected by FLMaV (lane 2), using the degenerate primers specific for the conserved phosphate 1 and 2 motifs of the closteroviral HSP70 gene (Tian *et al.*, 1996). Mk, DNA ladder; lane 3, extract from a symptomless fig seedling.



**Fig. 3.** Phylogenetic tree constructed with amino acid sequences of phosphate motifs 1 and 2 of FLMaV HSP70 protein and of members of the family *Closteroviridae* (abbreviations and accession numbers in parentheses). Genus *Crinivirus*: *Lettuce infectious yellows virus* (LIYV, U67448), *Tomato chlorosis virus* (ToCV, AJ968396), *Cucurbit yellow stunt disorder virus* (CYSDV, AJ223619), *Sweet potato chlorotic stunt virus* (SPCSV, AJ428554). Genus *Ampelovirus*: FLMaV-2 (AM286422), *Grapevine leafroll-associated virus 1* (GLRaV-1, AF195822), *Grapevine leafroll-associated virus 3* (GLRaV-3, U82937), *Grapevine leafroll-associated virus 4* (GLRaV-4, AF039553), *Grapevine leafroll-associated virus 5* (GLRaV-5, AF039552), *Pineapple mealybug wilt-associated virus 2* (PMWaV-2, AF414119), *Little cherry virus 2* (LCHV-2, AF531505). Genus *Closterovirus*: *Citrus tristeza virus* (CTV, NC001661), *Beet yellow stunt virus* (BSYV, U51931), *Beet yellows virus* (BYV, X73476), *Fig leaf mottling-associated virus* (FLMaV, AM113547), *Grapevine leafroll associated virus 2* (GLRaV-2, Y14131). HSP70 of Olive leaf yellowing-associated virus (OLYaV, AJ440010) was used as an outgroup.



**Fig. 4.** **A)** Closterovirus-like particles in a leaf dip from a symptomatic leaf of the *F. carica* accession F3. Bar = 100 nm. **B)** Heavily damaged phloem tissues showing necrotic and crushed cells with thickened cell walls (W). Aggregates of virus particles (V) fill the lumen of a cell with evident signs of plasmolysis and containing rows of small vesicles lining the plasmalemma (arrow heads). Inset shows a close-up of these vesicles and groups of cross-sectioned virus particles (arrow heads). Bars = 200 nm.



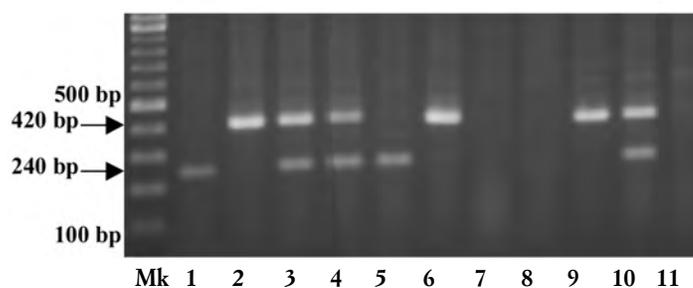
**Fig. 5.** Gel electrophoresis of a RT-PCR assay showing a product of *ca.* 360 bp amplified from total nucleic acid extracts of leaves from field-grown fig trees using F3-specific primers. Lane Mk, DNA ladder; lanes 1, 2, 4, and 7, F3-infected samples; lanes 3 and 6, negative samples; lane 5, F3 positive control; lane 8, water control.

HSP70 amino acid identity with comparable sequences of other members of the family *Closteroviridae* was *ca.* 48%, 37%, 36% and 28% for LChV-2, GLRaV-1, PMWaV-2 and FLMaV respectively.

A phylogenetic tree constructed with the HSP70 sequences of F3 and of other members of the family *Closteroviridae*, grouped F3 with members of the genus *Ampelovirus* and confirmed the allocation of FLMaV to the genus *Closterovirus* (Fig. 3).

**Electron microscopy.** Leaf dips from symptomatic F3 leaves showed filamentous particles with distinct cross banding, up to 2100 nm long and *ca.* 12 nm in diameter (Fig. 4A). Although there were no obvious outward signs of necrosis of the veins, large areas of the phloem were heavily damaged. Many sieve tubes had thickened walls, were necrotic and crushed (Fig. 4B). Some contained aggregates of filamentous virus particles that often filled the cell lumen (Fig. 4B) and membranous vesicles with fibrillar material. The presence of rows of vesicles lining the plasmalemma of some cells (Fig. 4B, inset) was a feature not previously observed in closterovirus-infected tissues (Martelli and Russo, 1984). In contrast to phloem, mesophyll cells had a normal appearance and a well preserved structure, and no filamentous virus particles or membrane-bound bodies typically associated with fig mosaic (Bradfute *et al.*, 1970; Plavsic and Milicic, 1980; Martelli *et al.*, 1993; Appiano *et al.*, 1995) were observed.

**Field survey and Multiplex-PCR.** In a survey conducted in Apulian (southern Italy) fig orchards, 4 of 57 samples from mosaic-diseased trees yielded a DNA fragment of the expected size when analysed by RT-PCR using F3-specific primers (Fig. 5). Multiplex-PCR assays proved effective in identifying mixed infections by F3 and FLMaV in field-grown trees (Fig. 6).



**Fig. 6.** Gel electrophoresis of a Multiplex-PCR assay showing single infections of field samples by F3 (240 bp amplicon, lanes 1 and 5) and by FLMaV (420 bp amplicon, lanes 2, 6 and 9). Lanes 3 and 4, natural mixed infection by F3-like and FLMaV of two field-grown fig trees; lane 10, artificial mixture of F3 and FLMaV cDNA; lanes 7 and 8, symptomless fig seedlings (negative controls); lane 11, water control.

The results of this study provide evidence that fig trees host an additional member of the family *Closteroviridae*, tentatively identified as a putative species of the genus *Ampelovirus*.

The similarity of symptoms shown by the Algerian fig accession in which the virus in question was first identified to those of plants infected by FLMaV (Elbeaino *et al.*, 2006) seems to justify the naming of this virus as Fig leaf mottle-associated virus 2 (FLMaV-2).

This designation would strongly suggest a change of the name of FLMaV to Fig leaf mottle associated virus 1 (FLMaV-1).

## ACKNOWLEDGEMENTS

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## REFERENCES

- Appiano A., Conti M. and Zini N., 1995. Cytopathological study of the double-membrane bodies occurring in fig plants affected by fig mosaic disease. *Acta Horticulturae* **386**: 585-592.
- Altschul S.F., Stephen F., Gish W., Miller W., Myers E.W., Lipman D.J., 1990. Basic local alignment search tool. *Journal of Molecular Biology*, **215**: 403-410.
- Bradfute O.R., Whitmoyer R.E., Nault R.L., 1970. Ultrastructure of plant leaf tissue infected with mite-borne viral-like particles. *Proceedings of the Electron Microscopy Society of America* **258**: 178-179.
- Condit I.J., Horne W.T., 1933. A mosaic of the fig in California. *Phytopathology* **23**: 887-896.
- Dodds J.A., 1993. dsRNA in diagnosis. In R.E.F. Mathews (ed). *Diagnosis of plant virus Diseases*, p. 273-294. CRC Press, Boca Raton, USA.
- Elbeaino T., Digiario M., De Stradis A., Martelli G.P., 2006.

- Partial characterization of a closterovirus associated with a chlorotic mottling of fig. *Journal of Plant Pathology*, **88**: 187-192.
- Felsenstein J., 1989. PHYLIP- phylogeny inference package (version 3.5). *Cladistics*, **5**: 164-166.
- Foissac X., Svanella-Dumas L., Gentit P., Dulucq M.J., Candresse T., 2001. Polyvalent detection of fruit tree Tricho, Capillo and Foveavirus by nested RT-PCR using degenerated and inosine containing primers (DOP RT-PCR). *Acta Horticulturae (ISHS)* **550**: 37-43.
- Marck C., 1988. "DNA Strider": a "C" programme for the fast analysis of DNA and protein sequences on the Apple Macintosh family computers. *Nucleic Acids Research* **16**: 1829-1836.
- Martelli G.P., Russo M., 1984. Use of thin sectioning for visualization and identification of plant viruses. *Methods in Virology* **8**: 143-224.
- Martelli G.P., Castellano M.A., Laforteza R., 1993. An ultrastructural study of fig mosaic. *Phytopathologia Mediterranea* **32**: 33-43.
- Pearson W.R., Lipman D.J., 1988. Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences USA* **85**: 2444-2448.
- Plavsic B., Milicic D., 1980. Intracellular changes in trees infected with fig mosaic. *Acta Horticulturae* **110**: 281-286.
- Saldarelli P., Minafra A., Martelli G.P., Walter B., 1994. Detection of grapevine leafroll- associated closterovirus III by molecular hybridization. *Plant Pathology* **43**: 91-96.
- Sambrook J., Fritsch E.F., Maniatis T., 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, USA.
- Tian T., Klaassen V.A., Soong J., Wisler G., Duffus J.E., Falk B.W., 1996. Generation of cDNAs specific to lettuce infectious yellows closterovirus and other whitefly-transmitted viruses by RT-PCR and degenerated oligonucleotide primers corresponding to the closterovirus gene encoding the heat shock protein 70 homologue. *Phytopathology* **86**: 1167-1172.
- Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G., 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876-4882.

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